

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 15/28, C12P 21/08, A61K 39/395, G01N 33/577		A2	(11) International Publication Number: WO 94/29351 (43) International Publication Date: 22 December 1994 (22.12.94)												
<p>(21) International Application Number: PCT/GB94/01290</p> <p>(22) International Filing Date: 15 June 1994 (15.06.94)</p> <p>(30) Priority Data:</p> <table> <tr><td>9312415.4</td><td>16 June 1993 (16.06.93)</td><td>GB</td></tr> <tr><td>9401597.1</td><td>27 January 1994 (27.01.94)</td><td>GB</td></tr> <tr><td>9402499.9</td><td>9 February 1994 (09.02.94)</td><td>GB</td></tr> <tr><td>9406244.5</td><td>29 March 1994 (29.03.94)</td><td>GB</td></tr> </table> <p>(71) Applicant (for all designated States except US): CELLTECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): MORGAN, Susan, Adrienne [GB/GB]; 1A Ennerdale Crescent, Slough, Berkshire SL1 6EH (GB). EMTAGE, John, Spencer [GB/GB]; 49 Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ (GB). BODMER, Mark, William [GB/GB]; Rose Cottage, 5 Manor Road, South Hinksey, Oxford OX1 5AS (GB). ATHWAL, Diljeet, Singh [GB/GB]; 33 Casella Road, New Cross Gate, London SE17 (GB).</p> <p>(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).</p>		9312415.4	16 June 1993 (16.06.93)	GB	9401597.1	27 January 1994 (27.01.94)	GB	9402499.9	9 February 1994 (09.02.94)	GB	9406244.5	29 March 1994 (29.03.94)	GB	<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
9312415.4	16 June 1993 (16.06.93)	GB													
9401597.1	27 January 1994 (27.01.94)	GB													
9402499.9	9 February 1994 (09.02.94)	GB													
9406244.5	29 March 1994 (29.03.94)	GB													
<p>(54) Title: ANTIBODIES</p> <p>(57) Abstract</p> <p>The invention provides antibodies with altered ability to fix complement. The invention further relates to pharmaceutical, therapeutic and diagnostic compositions containing said antibodies and to methods of therapy and diagnosis using said antibodies. The invention additionally provides a method of modulating the function of cell surface associated antigens using said antibodies. Also provided are processes for preparing said antibodies.</p>															

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

ANTIBODIES

FIELD OF THE INVENTION

- 5 This invention relates to altered antibodies, to pharmaceutical, therapeutic and diagnostic compositions containing said antibodies; to processes for preparing said compositions; to methods of therapy and diagnosis using said antibodies, to a method of modulating the function of cell surface associated antigens using said antibodies; to DNA sequences coding for
10 said antibodies; to cloning and expression vectors containing DNA sequences coding for said antibodies; to host cells transformed with said vectors and to processes for preparing said antibodies.

BACKGROUND OF THE INVENTION

- 15 In order for an antibody to be effective therapeutically it is desirable that it achieves the required physiological effect without producing any significant adverse toxic effects. Such toxic effects may be mediated, for example, via complement fixation.
- 20 Antibody when bound to its cognate antigen can link to and activate the complement cascade. Complement consists of a complex series of proteins. The proteins of the complement system form two interrelated enzyme cascades, termed the classical and alternative pathways, providing two routes to the cleavage of C3, the central event in the
25 complement system. The sequence of events comprising the classical complement pathway is recognition, enzymatic activation, and membrane attack leading to cell death. The recognition unit of the complement system is the C1 complex. The C1 complement protein complex is a unique feature of the classical complement cascade leading to C3
30 conversion. Complement fixation occurs when the C1q subcomponent binds directly to immunoglobulin antigen immune complex. Whether or not complement fixation occurs depends on a number of constraints. For example, only certain subclasses of immunoglobulin can fix complement even under optimal conditions. These are IgG1, IgG3 and IgM in man and
35 IgG2a, IgG2b and IgM in mice.

The C1q molecule is potentially multivalent for attachment to the complement fixation sites of immunoglobulin. The C_H2 domain of IgG and probably the C_H4 domain of IgM contain binding sites for C1q.

- 5 Fc bearing cells also play a role in enhancing the effect of the immune response by binding to and opsonising, phagocytosing or killing target cells coated with antibody of the relevant class. Three IgG binding receptors (Fc_yR) have been described for murine and human leukocytes. Fc_yRI has high binding affinity for monomeric IgG, while Fc_yRII and Fc_yRIII have low
- 10 affinity for mono IgG and interact mainly with antigen complexed IgG. The presence of Fc receptors confers on these immune cells the ability to mediate a number of effector mechanisms important in the effector phase of the humoral response.
- 15 The gamma 1 isotype of human IgG, like IgG3, binds to FcRI and, when complexed with its cognate antigen, activates complement and binds to FcRII and FcRIII. Conversely, human IgG2 and IgG4 are relatively inactive isotypes; both fail to activate the classical complement pathway and IgG4 binds weakly to FcRI [Burton, D R and Woof, J M (1992) *Adv. Immunol.* **51**, 1. Lucisano Valim, Y M and Lachmann, P J. (1991) *Clin. exp. Immunol.* **84**, 1].

- Localisation of amino acid residues of IgG that interact with FcRI in the C_H2 domain of human IgG is well established [Woof, J M *et al* (1986) *Molec. Immunol.* **23**, 319. Lund, J *et al* (1991) *J. Immunol.* **147**, 2657; Canfield, S M and Morrison, S L (1991), *J. exp. Med.* **173**, 1483; Chappel, S M *et al*, (1991) *Proc. Natl. Acad. Sci.* **88**, 9036; Chappel, S M *et al* (1993), *J. Biol. Chem.* **268**, 25124; Alegre, M-L *et al* (1992) *J. Immunol.* **148**, 3461]. Amino acid sequence comparisons of the C_H2 domains of antibodies from different species and subclasses that bind well to FcRI suggested that a region at the N-terminal end of C_H2 comprising residues Leu 234 - Ser 239 (using the Kabat Eu numbering system [Kabat, E A *et al* (1987) *Sequences of proteins of immunological interest. US Dept. of Health and Human Services, Bethesda, MD, USA*]) is critical for interaction with FcRI. The motif Leu 234, Leu 235, Gly 236, Gly 237, Pro 238, Ser 239, is present in all IgG isotypes with high affinity for FcRI [Woof, J M *et al*

- (1986), Molec. Immunol. 23, 319]. Domain exchanges between Ig's with different Fc effector functions have demonstrated the importance of C_H2 for FcRI binding [Canfield, S M and Morrison, S L (1991), J. exp. Med. 173, 1483; Chappel, S M *et al.* (1991) Proc. Natl. Acad. Sci. 88, 9036; Chappel, 5 S M *et al.* (1993), J. Biol. Chem. 268, 25124] in particular the residue 235. Replacement of the Leu residue at position 235 with a Glu residue reduces the affinity of IgG3 for FcRI by 100 fold [Lund, J *et al.* (1991) J. Immunol., 147, 2657; Canfield, S M and Morrison, S L (1991), J. exp. Med. 173, 1483]. The same Leu 235 to Glu change when performed on an IgG4 10 variant of OKT3 [Alegre, M-L *et al.* (1992) J. Immunol., 148, 3461] abolished its FcRI binding and, consequently, its mitogenic properties.

- Although the sequence requirements for FcRIII binding has been less extensively studied, Sarmay *et al.* [(1992) Molec. Immunol. 29, 633] have 15 identified the C_H2 domain residues 234 to 237 as important for IgG3 binding to all three Fc receptors. The relative importance of each residue differs with each Fc receptor with 235 and 237 being most important for FcRIII mediated cell killing.
- 20 In contrast, another Fc mediated function, C1q binding and subsequent complement activation, appears to require the carboxyl terminal half of the C_H2 domain [Tao, M H., Canfield, S M., and Morrison, S L(1991) J. Exp. Med. 173, 1025]. Morrison's group, following sequence analysis of polymorphisms in the C_H2 domain of human IgGs also identified the 25 importance of the C-terminal region of C_H2. With a Pro to Ser change at 331 in IgG1 they abolished complement fixation and reduced C1q binding [Tao, M H *et al.* (1993), J. Exp. Med. 178, 661]. Using inter- and intra-domain switch variants of CAMPATH-1, Greenwood *et al.* (1993) [Eur. J. Immunol. 23, 1098] further endorsed the importance of the C-terminal end 30 of C_H2. Complement fixation could be restored to human IgG4 with just the carboxyl terminal of C_H2 from residue 292 of IgG1 and not the N-terminal half or any other domain. Duncan & Winter (1988) [Nature, 332, 21] identified a motif in C_H2 of Glu 318, Lys 320 and Lys 322 of the mouse IgG2b isotype. Changing any of these residues abolished C1q binding, as 35 did the use of competitive peptides of sequences in this region. However, the C1q motif residues are also found in antibodies that do not fix

complement suggesting that these residues may well be necessary but not sufficient for complement activation.

- We have found that amino acid residues necessary for C1q and FcR binding of human IgG1 are located in the N-terminal region of the CH2 domain, residues 231 to 238, using a matched set of engineered antibodies based on the anti-HLA DR antibody L243. Changing the leucine 235 in the CH2 region of IgG3 and IgG4 to glutamic acid was already known to abolish FcRI binding, we have confirmed this for IgG1 and also found a concomitant abolition of human complement fixation with retention of FcRIII mediated function. Changing the glycine at 237 to alanine of IgG1 also abolished FcRI binding and reduced complement fixation and FcRIII mediated function. Exchanging the whole region 233 to 236, with the sequence found in human IgG2 abolished FcRI binding and complement fixation and reduced FcRIII mediated function of IgG1. In contrast, a change in the previously described C1q binding motif, from lysine at 320 to alanine had no effect on IgG1-mediated complement fixation.
- The proposed site Leu 234 - Leu 235 - Gly 236 - Gly 237 - Pro 238 - Ser 239, is present in all IgG isotypes with high affinity for FcγRI. Recent mutagenesis experiments on IgG3 antibodies have introduced point mutations in this region and the ability of the mutants to interact with FcγRI has been examined [Lund *et al* (1991) J. Immunol 147, 2657-2662]. The most marked effect is seen at position 235 where replacement of the naturally occurring Leu residue with a Glu residue produces an Ig with a >100-fold decrease in affinity for FcγRI.
- Our observation of the effect of this alteration at residue 235 on the ability of the antibody to fix complement was highly surprising. Earlier protein engineering studies had introduced mutations at various positions in order to locate the C1q-binding site on IgG [Duncan & Winter (1988) Nature, 332, 738-740]. The binding site for C1q was localised to three side chains, Glu 318, Lys 320 and Lys 322 of the mouse IgG2b isotype. Residues Glu 318, Lys 320 and Lys 322 are conserved in all the human IgGs, rat IgG2b and IgG2c, mouse IgG2a, IgG2b and IgG3, guinea pig IgG1 and rabbit IgG.

Further experiments showed that the affinity of human C1q for mutant mouse IgG2b antibodies in which residue 235 was mutated was unaffected i.e. it was in the same range of values as that obtained with the wild type.

- 5 Although the fact that altering residue 235 of the CH₂ region of IgG is known to abolish Fc_YRI binding as we too observed, this concomitant substantial reduction in complement fixation has not been reported or suggested elsewhere and was completely unexpected.

10 **SUMMARY OF THE INVENTION**

The invention provides a method of treating diseases in which antibody therapy leads to undesirable toxicity due to antibody mediated complement fixation comprising administering an altered antibody wherein one or more amino acid residues in the N-terminal region of the CH₂ domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody.

- 20 In a preferred embodiment the altered antibody binds to one or more cellular Fc receptors especially Fc_{RIII} and excluding Fc_{RI} i.e. the antibody does not bind significantly to Fc_{RI}, and more preferably binding to Fc_{RI} is abolished.

- 25 Accordingly in a further aspect the invention provides an altered antibody wherein one or more amino acid residues in the N-terminal region of the CH₂ domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered, as compared to unaltered antibody.

- 30 In a further preferred embodiment the invention therefore provides an altered antibody wherein one or more amino acid residues in the N-terminal region of the CH₂ domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody and said altered antibody binds to one or more cellular Fc receptors especially Fc_{RIII} and does not bind significantly to Fc_{RI}.

The constant region of the antibodies to be altered according to the invention may be of animal origin and is preferably of human origin. It may also be of any isotype but is preferably human IgG and most preferably human IgG1.

5

In a preferred embodiment of the invention the amino acid residue(s) which is altered lies within amino acid positions 231 to 239, preferably within 234 to 239.

10 In a particularly preferred embodiment of the invention the amino acid residue(s) which is altered lies within the motif Leu 234 Leu 235 Gly 236 Gly 237 Pro 238 Ser 239.

15 In a most preferred embodiment the amino acid residue(s) which is altered is either Leu 235 and/or Gly 237.

DETAILED DESCRIPTION OF THE INVENTION

As used herein the term 'altered' when used in conjunction with the ability of an antibody to fix complement most usually indicates a decrease in the 20 ability of antibody to fix complement compared to the starting antibody. By choosing appropriate amino acids to alter it is possible to produce an antibody the ability of which to fix complement is substantially reduced such as for example by altering residue Leu 235. It is also possible to produce an antibody with an intermediate ability to fix complement by, for example 25 altering amino acid residue Gly 237.

As used herein the phrase 'substantially reduce complement fixation' denotes that human complement fixation is preferably ≤30%, more 30 preferably ≤20% and most preferably ≤10% of the level seen with the starting wild type unaltered antibody.

The term 'significantly' as used with respect to FcRI binding denotes that the binding of antibody to FcRI is typically ≤20%, and is most preferably ≤10% of that seen with unaltered antibody.

The altered antibodies of the invention preferably bind to FcRIII as measured by their ability to mediate antibody dependent cellular cytotoxicity (ADCC) at a concentration no greater than ten times that of the wild type unaltered antibody.

5

The proteins encoded in the Major Histocompatibility Complex region of the genome are involved in many aspects of immunological recognition. It is known that all mammals and probably all vertebrates possess basically equivalent MHC systems and that immune response genes are linked to the MHC.

In man the major histocompatibility complex is the HLA gene cluster on chromosome 6. The main regions are D, B, C and A. The D region contains genes for class II proteins which are involved in cooperation and interaction between cells of the immune system. Many diseases have been found to be associated with the D region of the HLA gene cluster. Studies to date have shown associations with an enormous variety of diseases, including most autoimmune diseases (see for example, European Patent No. 68790). European Patent No. 68790 suggests controlling diseases associated with a particular allele of certain regions of the MHC such as the HLA-D region in humans by selectively suppressing the immune response(s) controlled by a monoclonal antibody specific for an MHC-class II antigen.

25 We have found that by altering an MHC-class II specific antibody at position 235 in the N-terminal region of the CH₂ domain it is possible to produce an antibody which fully retains its immunosuppressive properties but which has substantially reduced toxicity *in vitro* and is tolerated *in vivo*.

30 In a further preferred embodiment the invention provides an MHC specific antibody wherein one or more amino acid residues in the N-terminal region of the CH₂ domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody.

In a preferred embodiment the invention provides an MHC specific monoclonal antibody characterised in that said antibody has been altered at position 235 of the N-terminal region of the C_H2 domain.

5 In some instances such as with MHC specific monoclonal antibodies it may be desirable that the alteration in the N-terminal region of the C_H2 domain of the antibody while altering the ability to fix complement additionally inhibits the binding to FcRI receptors.

10 The antibodies are preferably specific for MHC-class II antigens and due to the alteration of one or more amino acid residues in the N-terminal region of the C_H2 domain will not bind significantly to FcRI.

15 In a further preferred embodiment the altered antibodies of the invention or for use according to the invention are directed against an MHC class II antigen characterised in that said antibody has been altered at position 235 of the N-terminal region of the C_H2 domain.

20 In a particularly preferred embodiment, the altered antibodies of the invention or for use according to the invention are directed against an MHC class II antigen characterised in that said antibody has been altered at position 235 of the N-terminal region of the C_H2 domain and the ability of said antibody to fix complement is altered as compared to unaltered antibody and said altered antibody binds to one or more cellular Fc receptors especially FcRIII and does not bind significantly to FcRI.

25 In a further aspect the invention provides a method for producing an altered antibody with altered ability to fix complement comprising altering one or more amino acids in the N-terminal region of the C_H2 domain of said antibody, altering the ability of said antibody to fix complement as compared with unaltered antibody.

30 As used herein the term 'altered antibody' is used to denote an antibody which differs from the wild type unaltered antibody at one or more amino acid residues in the N-terminal region of the C_H2 domain of the Fc region of the antibody. The alteration may for example comprise the substitution

or replacement of the starting wild type antibody amino acid by another amino acid, or the deletion of an amino acid residue.

5 The residue numbering used herein is according to the Eu index described
in Kabat *et al* [(1991) in: Sequences of Proteins of Immunological Interest,
5th Edition. United States Department of Health and Human Services.]

10 In human IgG1 and IgG3 antibodies the naturally occurring amino acid at position 235 of the N-terminal region of the CH₂ domain is a leucine residue. The alterations at position 235 of replacing leucine by glutamic acid or alanine have been found particularly effective at producing a potent immuno-suppressive antibody with minimal toxicity *in vitro* and which is tolerated *in vivo*.

15 15 The alteration at position 237 of replacing glycine by alanine has been found to produce an antibody with an intermediate ability to fix human complement. i.e. the complement fixation level is approximately 15-80%, preferably 20-60%, most preferably 20-40% of that seen with the starting wild type unaltered antibody.

20 20 The residue(s) could similarly be replaced using an analogous process to that described herein, by any other amino acid residue or amino acid derivative, having for example an inappropriate functionality on its side chain. This may be achieved by for example changing the charge and/or polarity of the side chain.

30 30 The altered antibodies of the invention may also be produced for example, by deleting residues such as 235, or by, for example, inserting a glycosylation site at a suitable position in the molecule. Such techniques are well known in the art, see for example the teaching of published European patent application EP-307434.

35 35 The altered antibodies of the invention may also be produced by exchanging lower hinge regions of antibodies of different isotypes. For example a G1/G2 lower hinge exchange abolished complement fixation and is a further preferred embodiment of the invention. This is described in

more detail in the accompanying examples. The G1/G2 lower hinge exchange results in an antibody with altered residues in the 231 to 238 region of the N-terminal region of the C_H2 domain wherein one or more residues may be altered and/or deleted.

5

In a particularly preferred embodiment of the invention the antibody is a human IgG1 antibody directed against an MHC class II antigen.

10

In a further aspect the invention provides a method of modulating the function of cell surface associated antigens avoiding complement mediated toxicity comprising administering an altered antibody wherein one or more amino acid residues in the N-terminal region of the C_H2 domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody.

15

In a preferred embodiment of this aspect of the invention said altered antibody is able to bind to one or more cellular Fc receptors especially FcRIII while binding to FcRI is significantly reduced.

20

Examples of such cell surface antigens include for example adhesion molecules, T-cell receptor, CD4, CD8, CD3, CD28, CD69, MHC Class I, MHC Class II and CD25.

25

The invention also includes therapeutic, pharmaceutical and diagnostic compositions comprising the altered antibodies according to the invention and the uses of these products and the compositions in therapy and diagnosis.

30

Thus in a further aspect the invention provides a therapeutic, pharmaceutical or diagnostic composition comprising an altered antibody according to the invention, in combination with a pharmaceutically acceptable excipient, diluent or carrier.

35

The invention also provides a process for the preparation of a therapeutic, pharmaceutical or diagnostic composition comprising admixing an altered

antibody according to the invention together with a pharmaceutically acceptable excipient, diluent or carrier.

5 The antibodies and compositions may be for administration in any appropriate form and amount according to the therapy in which they are employed.

10 The altered antibodies for use in the therapeutic, diagnostic, or pharmaceutical compositions, or for use in the method of treatment of diseases in which antibody therapy leads to undesirable toxicity due to antibody mediated complement fixation are preferably MHC specific antibodies most preferably specific for MHC Class II antigens, and most preferably have specificity for antigenic determinants dependent on the DR α chain.

15 The therapeutic, pharmaceutical or diagnostic composition may take any suitable form for administration, and, preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the product is for injection or 20 infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents.

25 Alternatively, the antibody or composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

If the antibody or composition is suitable for parenteral administration the formulation may contain, in addition to the active ingredient, additives such as: starch - e.g. potato, maize or wheat starch or cellulose - or starch 30 derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the formulation is for parenteral administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be 35 desirable to improve tolerance by formulating the antibody or compositions in a capsule which is insoluble in the gastric juices. It may also be

preferable to include the antibody or composition in a controlled release formulation.

If the antibody or composition is suitable for rectal administration the
5 formulation may contain a binding and/or lubricating agent, for example polymeric glycols, gelatins, cocoa-butter or other vegetable waxes or fats. The invention also provides methods of therapy and diagnosis comprising administering an effective amount of an altered antibody according to the invention to a human or animal subject.

10

The antibodies and compositions may be for administration in any appropriate form and amount according to the therapy in which they are employed. The dose at which the antibody is administered depends on the nature of the condition to be treated and on whether the antibody is being,
15 used prophylactically or to treat an existing condition. The dose will also be selected according to the age and conditions of the patient. A therapeutic dose of the antibodies according to the invention may be, for example, preferably between 0.1-25mg/kg body weight per single therapeutic dose and most preferably between 0.1-10mg/kg body weight per single therapeutic dose.
20

25

Immunological diseases which may be treated with the antibodies of the invention include for example joint disease such as ankylosing spondylitis, juvenile rheumatoid arthritis, rheumatoid arthritis; neurological disease such as multiple sclerosis; pancreatic disease such as diabetes, juvenile onset diabetes; gastrointestinal tract disease such as chronic active hepatitis, celiac disease, ulcerative colitis, Crohns disease, pernicious anaemia; skin diseases such as psoriasis; allergic diseases such as asthma and in transplantation related conditions such as graft versus host disease, and allograft rejection. Other diseases include those described in European Patent No. 68790.
30

35

The altered antibodies of the invention may also be useful in the treatment of infectious diseases e.g. viral or bacterial infections and in cancer immunotherapy.

As used herein the term 'antibody' is used to cover natural antibodies, chimeric antibodies and CDR-grafted or humanised antibodies. Chimeric antibodies are antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains 5 derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP 120694 (Celltech Limited), EP 125023 (Genentech Inc and City of Hope), EP 171496 (Res. Dev. Corp. Japan), EP 173494 (Stanford University) and WO 86/01533 (Celltech Ltd). CDR grafted or humanised antibodies are antibody molecules having 10 an antigen binding site derived from an immunoglobulin from a non-human species and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. Procedures for generating CDR-grafted or humanised antibodies are described in WO 91/09967 (Celltech Ltd), WO 90/07861 (Protein Design Labs. Inc) and WO 92/11383 (Celltech Ltd).

In further aspects the invention also includes DNA sequences coding for the altered antibodies according to the invention; cloning and expression vectors containing the DNA sequences, host cells transformed with the 20 DNA sequences and processes for producing the altered antibodies according to the invention comprising expressing the DNA sequences in the transformed host cells.

According to a further aspect of the invention there is provided a process 25 for producing an altered antibody of the invention which process comprises:

- a. producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain.
- 30 b. producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain.
- c. transfected a host cell with both operons, and
- d. culturing the transfected cell line to produce the antibody molecule

wherein at least one of the expression vectors contains a DNA sequence encoding an antibody heavy chain in which one or more amino acid residues in the N-terminal region of the C_H2 domain of said antibody has been altered from that in the corresponding unaltered antibody.

5

As will be readily apparent to one skilled in the art, the alteration in the N-terminal region of the C_H2 domain may be made using techniques such as site directed mutagenesis after the whole altered antibody has been expressed. To express unaltered antibody the DNA sequences should be

10 expressed following the teaching described above for altered antibody.

The DNA sequences preferably encode a humanised antibody; a CDR-grafted heavy and/or light chain or a chimeric antibody.

15 The cell line may be transfected with two vectors, the first vector containing the operon encoding the light chain-derived polypeptide and the second vector containing the operon encoding the heavy chain derived polypeptide. Preferably the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure
20 as far as possible that each polypeptide chain is equally expressed.

Alternatively, a single vector may be used, the vector including a selectable marker and the operons encoding both light chain- and heavy chain-derived polypeptides.

25

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known *per se*. Such methods are shown, for instance, in Maniatis *et al*, Molecular Cloning, Cold Spring Harbor, New York 1989 and Primrose and Old, Principles of Gene
30 Manipulation, Blackwell, Oxford, 1980.

The altered antibody according to the invention is preferably derived from the anti-MHC antibody L243, which has been deposited at the American Type Culture Collection, Rockville, Maryland USA under Accession number

35 ATCC HB55, and is most preferably a chimeric or a CDR-grafted derivative

thereof. L243 was previously described by Lampson and Levy [J. Immunol. (1980) 125, 293].

- The standard techniques of molecular biology may be used to prepare
5 DNA sequences coding for the altered antibodies according to the invention. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. See for example "PCR Technology Principles and
10 Applications for DNA Amplification" (1989), Ed. H. A. Erlich, Stockton Press, N.Y. London. For example, oligonucleotide directed synthesis as described by Jones *et al* [Nature, 321, 522 (1986)] may be used. Also oligonucleotide directed mutagenesis may be used as described by Kramer *et al* [Nucleic Acid Res. 12 9441 (1984)].
15 Any suitable host cell/vector system may be used for the expression of the DNA sequences coding for the altered antibody. Bacterial e.g. *E.coli* and other microbial systems may be used. Eucaryotic e.g. mammalian host cell expression systems may also be used such as for example COS cells
20 and CHO cells [Bebbington, C R (1991) Methods 2, 136-145], and myeloma or hybridoma cell lines [Bebbington, C R *et al* (1992) Bio/Technology 10, 169-175].
25 Where the altered antibody is derived from L243 CHO based expression systems are preferably used.
Assays for determining FcRIII binding indirectly via ADCC assays and for determining complement fixation and C1q binding are well known in the art, and are described in detail in the following examples.
30 Immune function/immunosuppression by antibodies may be assayed using techniques well known in the art including for example: Mixed Lymphocyte Responses and T-cell antigen recall responses to Tetanus Toxoid. These assays are described in detail in the following examples.
35

The invention is illustrated in the following non-limiting examples and with reference to the following figures in which:

Figure 1 shows: a map of plasmid pMR15.1

Figure 2 shows: a map of plasmid pMR14

5 Figure 3 shows: the nucleotide sequence and predicted amino acid sequence of L243 heavy chain

Figure 4 shows: the nucleotide and amino acid sequences of
(a) clone 43, (b) clone 183 (c) clone 192

10 Figure 5 shows: the nucleotide sequence and predicted amino acid sequence of L243 light chain

Figure 6 shows: a map of plasmid pGamma 1

Figure 7 shows: a map of plasmid pGamma 2

Figure 8 shows: the nucleotide sequence of hinge and C_H2 region of human C-gamma 1

15 Figure 9 shows: Antigen binding potency of L243 human isotype series

- | | | | |
|---|------------|---|------------|
| + | G1 | + | G4 [L235E] |
| - | G1 [L235A] | - | G4 |
| X | G1 [G237A] | V | 100% |
| ◆ | G1 [K320A] | | |

20 Figure 10 shows: FcRI binding of L243 isotype series

- | | | | |
|---|------------|---|---------------|
| + | G1 | ▼ | G4 |
| ◆ | G1 [G237A] | X | G1 [L235A] |
| ● | G2 | X | G1/G2-L-hinge |
| - | G1 [L235E] | △ | G4 [L235E] |
| + | G1 [K320A] | | |

25 Figure 11 shows: human complement fixation by L243 isotype series

- | | | | |
|---|-------------|---|---------------|
| + | G1 | ▼ | G4 |
| ◆ | G1 [G 237A] | X | G1 [L235A] |
| ● | G2 | X | G1/G2 L-hinge |
| - | G1 [L235E] | △ | G4 [L235E] |
| + | G1 [K320A] | | |

30 Figure 12 shows: binding of human Clq to L243 human isotype series

- | | | | |
|---|------------|---|-------------|
| + | G1 | ● | Cells alone |
| X | G4 [L235E] | ▼ | Cells + C1q |
| X | G1 [L235E] | ◆ | G1 [K320A] |
| + | G4 | | |

35

Figure 13 shows: human complement fixation by L243 isotype

- | | | | |
|---|------------|---|------------|
| + | G1 | + | G1 [L235A] |
| ▲ | G2 | ● | G4 [L235E] |
| ■ | G1 [L235E] | ◆ | G1 [K320A] |
| ✗ | G4 | | |

5

Figure 14 shows: guinea pig complement fixation by L243 isotype

- | | | | |
|---|------------|---|------------|
| + | G1 | + | G1 [L235A] |
| ▲ | G2 | ● | G4 [L235E] |
| ■ | G1 [L235E] | ◆ | G1 [K320A] |
| ✗ | G4 | | |

10

Figure 15 shows: rabbit complement fixation by L243 isotype

- | | | | |
|---|------------|---|------------|
| + | G1 | + | G1 [L235A] |
| ▲ | G2 | ● | G4 [L235E] |
| ■ | G1 [L235E] | ◆ | G1 [K320A] |
| ✗ | G4 | | |

15

Figure 16 shows: FcRIII binding of L243 isotype series by ADCC

- | | | | |
|---|------------|---|---------------|
| + | G1 | ■ | G4 |
| ◆ | G1 [K320A] | + | G1 [G237A] |
| ● | G2 | ✗ | G1/G2 L-hinge |
| ■ | G1 [L235A] | ✗ | G4 [L235E] |
| ▲ | G1 [L235E] | | |

20

Figure 17 shows: L243 Isotype Series Inhibition of TT recall response

- | | |
|---|----------------|
| ■ | G1 |
| * | G2 |
| □ | G4 |
| △ | cyclosporin |
| ✗ | medium control |

25

Figure 18 shows: L243 Isotype Series Inhibition of TT recall response

30

- | | | | |
|---|----------------|---|---------------|
| ■ | hG1 | * | G1/G2 L-hinge |
| + | hG1 [L235E] | □ | G4 [L235E] |
| ◊ | medium control | | |
| △ | cyclosporin | | |

35

Figure 19 shows: L243 Isotype Series Inhibition of Mixed Lymphocyte Reaction.

- | | | | |
|---|-------------|---|---------------|
| ■ | hG1 | * | G1/G2 L-hinge |
| + | hG1 [L235E] | □ | G4 [L235E] |

- ◇ cyclosporin
- △ medium control

Figure 20 shows: L243 Isotype Series Inhibition of TT response

5

- G1
- + G1 [L235A]
- * G1 [G237A]
- ◇ Cyclosporin
- △ Medium control

Figure 21 shows: L243 Isotype Series Inhibition of Mixed Lymphocyte Reaction

10

- G1 [L235E]
- + G1 [L235A]
- G1
- ◇ Cyclosporin
- △ Medium control

Figure 22 shows: the nucleotide and amino acid sequence of VI region in L243-gL1

15

Figure 23 shows: shows the nucleotide and amino acid sequence of VI region of

20

L243-gL2

Figure 24 shows: the nucleotide and amino acid sequence of Vh region of L243-gH

25

Figure 25 shows: a graph of the results of a competition assay for L243 grafts vs FITC-chimeric L243

- cH cL
- + cH gL1
- * gH cL
- gH gL1

Figure 26 shows: a graph of a Scatchard analysis for L243 gamma 4

30

- | | |
|----------|------------|
| ■ cH cL | Kd = 4.1nM |
| + gH gL1 | Kd = 6.4nM |
| * gH gL2 | Kd = 9.6nM |

Figure 27 shows: a graph of FcRIII binding of chimeric, grafted and grafted [L235E] L243 as measured by ADCC

35

- Chimeric G1 wt
- + Chimeric G1 [L235E]

—*— Graft G1 wt

—□— Graft G1 [L235E]

Figure 28 shows: a graph of immunosuppressive activity of CDR grafted L243 measured by MLR

5 —■— Graft G1 wt

—+— Graft G1 [L235E]

* Cyclosporin

—*— Chimeric G1 wt

—◇— Chimeric G1 [L235E]

✗ Medium Control

10 Figure 29 shows: a graph of CDR grafted L243 and grafted [L235E] L243 TT recall response

—■— Graft G1 wt

—+— Graft G1 [L235E]

* Cyclosporin

—*— Chimeric G1 wt

—◇— Chimeric G1 [L235E]

✗ Medium Control

15 Figure 30 shows: a graph of complement mediated cytotoxic potency of CDR grafted L243 and CDR grafted [L235E] L243

—■— Chimeric G1 wt

—+— Chimeric G1 [L235E]

* Graft G1 wt

—□— Graft G1 [L235E]

25

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

30 EXAMPLES

Example 1

Gene Cloning and Expression

RNA preparation from L243 hybridoma cells

Total RNA was prepared from $3 \times 10^{exp} 7$ L243 hybridoma cells as described below. Cells were washed in physiological saline and dissolved in RNAzol (0.2ml per $10^{exp} 6$ cells). Chloroform (0.2ml per 2ml

homogenate) was added, the mixture shaken vigorously for 15 seconds and then left on ice for 15 minutes. The resulting aqueous and organic phases were separated by centrifugation for 15 minutes in an Eppendorf centrifuge and RNA precipitated from the aqueous phase by the addition of 5 an equal volume of isopropanol. After 15 minutes on ice, the RNA was pelleted by centrifugation, washed with 70% ethanol, dried and dissolved in sterile, RNAase free water. The yield of RNA was 350 µg.

Amino acid sequence of the L243 light chain.

10 The sequence of the first nine amino acids of the mature L243 light chain was determined to be NH₂-DIQMTQSPAS.

PCR cloning of L243 Vh and VL

15 The cDNA genes for the variable regions of L243 heavy and light chains were synthesised using reverse transcriptase to produce single stranded cDNA copies of the mRNA present in the total RNA, followed by Polymerase Chain Reaction (PCR) on the cDNAs with specific oligonucleotide primers.

20 a) cDNA synthesis

cDNA was synthesised in a 20µl reaction containing the following reagents: 50mM Tris-HCl PH8.3, 75mM KCl, 10mM dithiothreitol, 3mM MgCl₂, 0.5mM each deoxyribonucleoside triphosphates, 20 units RNAsin, 75ng random hexanucleotide primer, 2µg L243 RNA and 200 units Moloney Murine Leukemia Virus reverse transcriptase. After incubation at 42°C for 60 min the reaction was terminated by heating at 95°C for 5 minutes.

b) PCR

30 Aliquots of the cDNA were subjected to PCR using combinations of primers for the heavy and light chains. The nucleotide sequences of the 5' primers for the heavy and light chains are shown in Tables 1 and 2 respectively. These sequences, all of which contain a restriction site starting 6 nucleotides from their 5' ends, followed by 35 the sequence GCCGCCACC to allow optimal translation of the resulting mRNAs, an initiator codon and a further 20 - 30

nucleotides, are a compilation based on the leader peptide sequences of known mouse antibodies [Kabat *et al* (1991) in Sequences of Proteins of Immunological Interest, 5th Edition - United States Department of Health and Human Services].

5

The 3' primers are shown in Table 3. The light chain primer spans the V - C junction of the antibody and contains a restriction site for the enzyme SphI to facilitate cloning of the VI PCR fragment. The heavy chain 3' primers are a mixture designed to span the J - C junction of the antibody. The first 23 nucleotides are identical to those found at the start of human C - gamma 1, 2, 3 and 4 genes and include the ApaI restriction site common to these human isotypes. The 3' region of the primers contain a mixed sequence based on those found in known mouse antibodies [Kabat E A, Wu, T.T.; Perry H M, Gottesman K S, and Foeller L; In: Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services (1991)].

20

The combinations of primers described above enables the PCR products for Vh and VI to be cloned directly into the appropriate expression vector (see below) to produce chimeric (mouse - human) heavy and light chains and for these genes to be expressed in mammalian cells to produce chimeric antibodies of the desired isotype.

25

Incubations (20 µl) for the PCR were set up as follows. Each reaction contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 1 - 6 pmoles 5' primer mix (Table 4), 6 pmoles 3' primer, 1 µl cDNA and 0.25 units Taq polymerase. Reactions were incubated at 95°C for 5 minutes and then cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, aliquots of each reaction were analysed by electrophoresis on an agarose gel. Reactions containing 5' primer mixes B1, B2, B3 and B5 produced bands with sizes consistent with full length VI fragments while reaction B9 produced a fragment with a size

expected of a Vh gene. The band produced by the B1 primers was not followed up as previous results had shown that this band corresponds to a light chain pseudogene produced by the hybridoma cell.

5

c) Molecular cloning of the PCR fragments

DNA fragments produced in reactions B2, B3 and B5 were digested with the enzymes BstB1 and S_pI, concentrated by ethanol precipitation, electrophoresed on a 1.4 % agarose gel and DNA bands in the range of 400 base pairs recovered. These were cloned by ligation into the vector pMR15.1 (Figure 1) that had been restricted with BstB1 and S_pI. After ligation, mixtures were transformed into E. coli LM1035 and plasmids from the resulting bacterial colonies screened for inserts by digestion with BstB1 and S_pI. Representatives with inserts from each ligation were analysed further by nucleotide sequencing.

20

In a similar manner, the DNA fragments produced in reaction B9 and digested with HindIII and ApaI were cloned into the vector pMR14 (Figure 2) that had been restricted with HindIII and ApaI. Again, representative plasmids containing inserts were analysed by nucleotide sequencing.

d) Nucleotide sequence analysis

25

Plasmid DNA (pE1701 and pE1702) from two isolates containing Vh inserts from reaction B9 was sequenced using the primers R1053 (which primes in the 3' region of the HCMV promoter in pMR14) and R720 (which primes in the 5' region of human C - gamma 4 and allows sequencing through the DNA insert on pMR14). The determined nucleotide sequence and predicted amino acid sequence of L243 Vh in pE1702 is given in Figure 3. The nucleotide sequence for the Vh insert in pE1701 was found to be identical to that in pE1702 except at nucleotide 20 (A in pE1701) and nucleotide 426 (A in pE1701). These two differences are in the signal peptide and J regions of Vh respectively and indicate that the two clones

30

35

examined are independent isolates arising from the use of different primers from the mixture of oligonucleotides during the PCR stage.

To analyse the light chain clones, sequence derived from priming with R1053 was examined. The nucleotide sequence and predicted amino acid sequence of the VI genes arising from reactions B2 (clone 183), B3 (clone 43) and B5 (clone 192) are shown in Figure 4. Comparison of the predicted protein sequences shows the following:

10

i) clones 182, 183, 43 and 45 all code for a VI gene which, when the signal peptide is removed, produces a light chain whose sequence is identical to that determined by amino acid sequence analysis for L243 light chain (see above).

15

ii) clones 182 and 183 contain a VI gene that codes for a signal peptide of 20 amino acids, while the VI gene in clones 43 and 45 results from priming with a different set of oligonucleotides and has a leader sequence of only 15 amino acids.

20

iii) Clone 192 does not code for L243 VI. Instead, examination of the database of antibody sequences [Kabat, 1991] indicates that clone 192 contains the VI gene for MOPC21, a light chain synthesised by the NS1 myeloma fusion partner used in the production of the L243 hybridoma.

25

iv) Clones 182 and 183 are identical except at nucleotide 26 (T in clone 182, C in clone 183). This difference can be accounted for by the use of different primers in the PCR and indicates that clones 182 and 183 are independent isolates of the same gene. The nucleotide sequence and predicted amino acid sequence of the complete VI gene from clone 183 is shown in Figure 5.

Construction of human gamma 1 and gamma 2 isotypes.

35

The L243 Vh gene was subcloned on a HindIII - ApaI fragment into pGamma 1 and pGamma 2, vectors containing the human C - gamma 1 and C - gamma 2 genes respectively (Figures 6 and 7).

Human Isotype mutants

PCR mutagenesis was used to change residue 235 in human C - gamma 1 contained in the vector pGamma 1 from leucine to either glutamic acid or to

5 alanine and to change residue 237 from glycine to alanine. The lower hinge region of human C-gamma 1 was also replaced by the corresponding region of human C-gamma 2. The following oligonucleotides were used to effect these changes:

10 i) L235E change

R4911 5' GCACCTGAACTCGAGGGGGACCGTCAGTC3'

R4910 5'CCCCCCTCGAGTTCAGGTGCTGAGGAAG3'

ii) L235A change

15 R5081 5'GCACCTGAACTCGCAGGGGGACCGTCAGTC3'

R5082 5'GACTGACGGTCCCCCTGCGAGTTCAGGTGC3'

III) G237A change

R5088 5'GCACCTGAACTCCTGGGTGCACCGTCAGTC3'

20 R5087 5'GACTGACGGTGCACCCAGGAGTTCAGGTGC3'

IV) Exchange of lower hinge regions

R4909 5'GCACCTCCAGTGGCAGGACCGTCAGTCTTCCTC3'

R4908 5'CGGTCTGCCACTGGAGGTGCTGAGGAAGAG3'

25

Other oligonucleotides used in the PCR mutagenesis are:

R4732 5'CAGCTCGGACACCTCTCTCCTCC3'

R4912 5'CCACCACCACGCATGTGACC3'

30

R4732 and R4912 prime between nucleotides 834 and 858 and between nucleotides 1156 and 1137 respectively in human C - gamma 1 (Figure 8).

35

The general strategy for the PCR mutagenesis was as follows. For each amino acid change, two rounds of PCR were used to generate DNA fragments containing the required substitutions. These fragments were

then restricted with the enzymes Bgl II and Sty1 and used to replace the corresponding fragments containing the wild type sequence in the pGamma 1 vector, (Figure 6).

- 5 For the first round PCR, reactions (20 µl) were prepared containing the following reagents : 10 mM Tris - HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 50 ng pGamma 1 DNA, 0.4 unit Taq polymerase and 6 pmoles of each of the primer. The following combinations of primers were used:

10

R4911 / R4912,

R4910 / R4732,

R5081 / R4912,

R5082 / R4732,

15

R5088 / R4912,

R5087 / R4732,

R4909 / R4912,

R4908 / R4732.

- 20 After 30 cycles through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, the reactions were extracted with chloroform, the newly synthesised DNA precipitated with ethanol, dissolved in water and electrophoresed on a 1.4 % agarose gel. Gel slices containing the DNA fragments were excised from the gel, the DNA recovered from the agarose 25 using a "Mermaid"™ kit (from Stratech Scientific Ltd., Luton, England) and eluted into 20µl sterile water.

- Second round PCR was in a 100 µl reaction containing 10 mM Tris - HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01 % gelatin, 0.25 mM each 30 deoxyribonucleoside triphosphate, 2 units Taq polymerase, 1/20 of each pair of DNA fragments from the first round reaction and 30 pmol of each of R4732 and R4912. After 30 cycles, see above, the reactions were extracted with phenol / chloroform (1/1) and precipitated with ethanol. Fragments were digested with BglII and Sty1, electrophoresed on a 1.4 % 35 agarose gel and DNA bands of 250 base-pairs recovered from gel slices as previously described.

These Bgl II - Sty1 fragments were ligated in a 3 - way ligation to the 830 base-pair Sty1 - EcoR1 fragment, containing the C - terminal part of the CH₂ domain and the entire CH₃ domain of human C - gamma 1, and the

- 5 BglIII - EcoR1 vector fragment from pGamma1 (see Figure 6). After transformation into LM1035, plasmid minipreps from resulting colonies were screened for the presence of the Bgl II - Sty1 fragment and representatives of each taken for nucleotide sequence analysis. From this, plasmids containing the desired sequence were identified and, for future reference, named as follows :

pGamma1[L235E] containing glutamic acid at residue 235,

pGamma1[L235A] containing alanine at residue 235,

pGamma1[G237A] containing alanine at residue 237,

- 15 pGamma1 [g1→g2] containing the C-gamma 2 lower hinge region.

The above plasmids were each restricted with Hind111 and Apa1 and the Hind111 - Apa1 fragment containing L243 Vh inserted to produce the following plasmids:

- 20 L243Gamma1[L235E]

L243Gamma1[L235A]

L243Gamma1[G237A]

L243Gamma [g1→g2]

- 25 a) Production of chimeric L243 antibody

Antibody for biological evaluation was produced by transient expression of the appropriate heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation.

- 30

On the day prior to transfection, semi - confluent flasks of CHO-L761 cells were trypsinised, the cells counted and T75 flasks set up each with 10exp7 cells.

- 35 On the next day, the culture medium was changed 3 hours before transfection. For transfection, the calcium phosphate precipitate was

prepared by mixing 1.25 ml of 0.25M CaCl₂ containing 50 µg of each of heavy and light chain expression vectors with 1.25 ml of 2xHBS (16.36 gm NaCl, 11.9 gm HEPES and 0.4 gm Na₂HPO₄ in 1 litre water with the pH adjusted to 7.1 with NaOH) and adding immediately into the medium on the

5 cells. After 3 hours at 37 °C in a CO₂ incubator, the medium and precipitate were removed and the cells shocked by the addition of 15 ml 15 % glycerol in phosphate buffered saline (PBS) for 1 minute. The glycerol was removed, the cells washed once with PBS and incubated for 48 - 96 hours in 25 ml medium containing 10 mM sodium butyrate. Antibody was

10 purified from the culture medium by binding to and elution from protein A - Sepharose and quantitated using an Ig ELISA (see below).

b) ELISA

For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a

15 F(ab)2 fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson Immuno-research, code 109-006-098) at 5 µg/ml in coating buffer (15mM sodium carbonate, 35mM sodium hydrogen carbonate, pH6.9). Uncoated antibody was removed by washing 5 times with distilled water. Samples and purified standards to be quantitated were

20 diluted to approximately 1 µg/ml in conjugate buffer (0.1M Tris-HCl pH7.0, 0.1M NaCl, 0.2% v/v Tween 20, 0.2% w/v Hammersten casein). The samples were titrated in the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the plates incubated at room temperature for 1 hr with shaking. After the first incubation step the plates

25 were washed 10 times with distilled water and then incubated for 1 hr as before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate buffer. The plate was washed again and substrate solution (0.1 ml) added to each well. Substrate solution contained

30 150 µl N,N,N,N-tetramethylbenzidine (10 mg/ml in DMSO), 150 µl hydrogen peroxide (30% solution) in 10 ml 0.1M sodium acetate/sodium citrate, pH6.0. The plate was developed for 5 -10 minutes until the absorbance at 630nm was approximately 1.0 for the top standard. Absorbance at 630nm was measured using a plate reader and the

35 concentration of the sample determined by comparing the titration curves with those of the standard.

TABLE 15 Oligonucleotide primers for the 5' region
of mouse heavy chains.

- CH1 : 5'ATGAAATGCAGCTGGTCAT(G,C)TTCTT3'
- 10 CH2 : 5'ATGGGATGGAGCT(A,G)TATCAT(C,G)(C,T)TCTT3'
- CH3 : 5'ATGAAG(A,T)TGTGGTAAACTGGGTTT3'
- 15 CH4 : 5'ATG(G,A)ACTTTGGG(T,C)TCAGCTTG(G,A)T3'
- CH5 : 5'ATGGACTCCAGGCTCAATTAGTTT3'
- CH6 : 5'ATGGCTGTC(C,T)T(G,A)G(G,C)GCT(G,A)CTCTTCTG3'
- 20 CH7 : 5'ATGG(G,A)ATGGAGC(G,T)GG(G,A)TCTTT(A,C)TCTT3'
- CH8 : 5'ATGAGAGTGCTGATTCTTTGTG3'
- 25 CH9 : 5'ATGG(C,A)TTGGGTGTGGA(A,C)CTTGCTATT3'
- CH10 : 5'ATGGGCAGACTTACATTCTCATTCCCT3'
- CH11 : 5'ATGGATTTGGGCTGATTTTTTATTG3'
- 30 CH12 : 5'ATGATGGTGTAAAGTCTCTGTACCT3'

Each of the above primers has the sequence
5'GCGCGCAAGCTGCCGCCACC3' added to its 5' end.

TABLE 2Oligonucleotide primers for the 5' region of
mouse light chains.

5

CL1 : 5'ATGAAGTTGCCTGTTAGGCTGTTGGTGCT3'

10

CL2 : 5'ATGGAG(T,A)CAGACACACTCCTG(T,C)TATGGGT3'

CL3 : 5'ATGAGTGTGCTCACTCAGGTCCCT3'

CL4 : 5'ATGAGG(G,A)CCCCTGCTCAG(A,T)TT(C,T)TTGG3'

15

CL5 : 5'ATGGATTT(T,A)CAGGTGCAGATT(T,A)TCAGCTT3'

CL6 : 5'ATGAGGT(T,G)C(T,C)(T,C)TG(T,C)T(G,C)AG(T,C)T(T,C)CTG
(A,G)G3'

20

CL7 : 5'ATGGGC(T,A)TCAAGATGGAGTCACA3'

CL8 : 5'ATGTGGGGA(T,C)CT(G,T)TTT(T,C)C(A,C)(A,C)TTTTCA
AT3'

25

CL9 : 5'ATGGT(G,A)TCC(T,A)CA(G,C)CTCAGTTCCCTT3'

CL10 : 5'ATGTATATATGTTGTTGTCTATTC3'

30

CL11 : 5'ATGGAAGCCCCAGCTCAGCTTCTCTT3'

Each of the above primers has the sequence
5'GGACTGTTCGAAGGCCGCCACC3' added to its 5' end.

TABLE 3Oligonucleotide primers for the 3' ends
of mouse Vh and VI genes.

5 Light chain (CL12):

5'GGATACAGTTGGTGCAGCATCCGTACGTT3'

10

Heavy chain (R2155):

5'GCAGATGGGCCCTCGTTGAGGCTG(A,C)(A,G)GAGAC(G,T,A)GTGA3'

15

TABLE 45' Primer mixtures for PCR

20

B1 : CL2.

B2 : CL6.

B3 : CL8.

25 B4 : CL4, CL9.

B5 : CL1, CL3, CL5, CL7, CL10, CL11.

B6 : CH6.

B7 : CH7.

B8 : CH2, CH4.

30 B9 : CH1, CH3, CH5, CH8, CH9, CH10, CH11, CH12.

Example 2**Biological properties of engineered L243**

The aim of the following experiments was to separate the immunosuppressive effects of anti-MHC-II antibodies from possible toxic

- 5 consequences of their use. In the process we hope to demonstrate which Fc effector functions are necessary for immunosuppression.

ANTIGEN BINDING POTENCY BY INHIBITION ASSAY

The principle of this experiment is that antibodies that have the same

- 10 binding will compete equally well with a labelled antibody for their cognate antigen. Any changes in the antigen binding potency of the engineered L243 antibodies will be revealed in this system.

Murine L243 (IgG2a) was labelled with fluorescein (FITC) using standard.

- 15 techniques. All dilutions, manipulations and incubations were done in Phosphate Buffered Saline (Gibco UK) containing 0.1% Sodium Azide (BDH UK) and 5% Foetal Calf Serum (Sigma UK). Serial dilutions of engineered antibodies in 100µl in RB polystyrene tubes (2052 12x75mm Falcon UK) were premixed with a constant amount in 100µl (at a previously
20 determined optimal concentration) of the labelled antibody on 5x10⁴ indicator cells (JY B lymphoblastoid cell line bearing high levels of HLA-DR). Cells and antibody were incubated together at 4°C for 30min, washed twice and binding revealed using a Fluorescence Activated Cell Scanner (FACS Becton Dickinson). After appropriate analysis, median
25 fluorescence intensity is plotted against antibody concentration.

Results

As expected, none of the changes in the Fc portion of the molecule affected antigen binding ability (Figure 9).

30

ASSESSMENT OF FCγ RI BINDING.

The ability of the engineered variants of L243 to bind to FcgRI was measured. The principle of this experiment is that antibodies will bind to

35 cells through Fc receptors and the affinity of this interaction is determined by the subclass and hence the structure of the Fc of the antibody. The

assay is based on the ability of the engineered antibodies to compete for binding with FITC labelled murine IgG2a to IFN γ stimulated U937 cells.

- U937 (myelomonocytic) cells, when incubated with 500 μ M IFN γ 5 (Genzyme UK) for 24 hours, expresses high levels of FcgRI, as assessed by CD64 binding and monomeric IgG2a binding, low levels of Fc γ RII and no Fc γ RIII.
- U937 cells are washed extensively in DMEM containing 25mM HEPES 10 (Gibco UK), incubated for 2 hours at 37°C in RPMI 1640 (Gibco UK) and then washed again in DMEM containing 25mM HEPES (Gibco UK) to remove bovine IgG bound to Fc receptors. Serial dilutions of engineered antibodies were prepared in 50 μ l in Phosphate Buffered Saline (Gibco UK) 15 containing 0.1% sodium azide in V-bottom 96 well microtitre plates' (ICN/Flow UK) and were incubated with 5x10⁴ U937 cells in 50 μ l for 30min at 4°C. 50 μ l of FITC labelled IgG2a antibody was then added to all wells, at a previously determined optimal concentration, for a further 90min at 4°C. Cells were washed once in the microtitre tray, transferred to RB polystyrene tubes (2052 12x75mm Falcon UK) washed once again and 20 binding was revealed using a Fluorescence Activated Cell Scanner (FACS Becton Dickinson). After appropriate analysis, median fluorescence intensity is plotted against antibody concentration.

Results

- Changes in the N-terminal region of the CH2 domain of IgG1 had profound effects on binding to FcRI (Figure 10). As expected, wild type IgG1 bound well to FcRI, IgG4 about 10 times less well and IgG2 did not bind at all. We have confirmed that the Leu 235 to Glu change in human IgG4 reduced its low FcRI binding to nothing and that the same change in IgG1 30 completely abolishes FcRI binding. Ala at 235 reduced (by about 100 fold) but did not ablate FcRI binding. Changing Gly 237 to Ala of IgG1 also abolished FcRI as did exchanging the whole region 233 to 236, with the sequence found in human IgG2. The G1[K320A] change had no effect on FcRI binding.

**ANTIBODY DEPENDENT COMPLEMENT
MEDIATED CYTOTOXICITY.**

5 The ability of the engineered variants of L243 to fix human complement
was assessed using the technique of antibody dependent complement
mediated cytotoxicity.

10 The principle of the experiment is that antibodies will mediate complement
lysis of target cells bearing their cognate antigen if the Fc of the antibody is
able to interact with the components of the (usually classical) complement
cascade. The critical interaction is with the C1q molecule.

15 The source of complement in these experiments is human venous blood
freshly drawn into endotoxin free glass bottles which is then allowed to clot
at 37°C for 1 hour. The clot is detached from the glass and then incubated
at 4°C for 2 hours to allow it to retract. The clot is then removed and the
serum separated from the remaining red cells by centrifugation at 1000g.
Once prepared, the serum can be stored for up to one month at -20°C
without noticeable deterioration of potency but is best used fresh.

20 20 All manipulations, dilutions and incubations are done in RPMI 1640
medium (Gibco UK) containing 2mM Glutamine (Gibco UK) and 10% foetal
calf serum (Sigma UK). Target cells (JY B lymphoblastoid cell line bearing
high levels of HLA-DR) are labelled with 1mCi Na⁵¹Cr for 1 hour at room
25 temperature, agitated every 15 min. The cells are then washed three
times, to remove free radiolabel, and resuspended at 2x10⁶/ml. Serial
antibody dilutions are prepared in duplicate in V-bottom 96 well microtitre
plates (ICN/Flow UK) in 25µl. Control wells containing medium only are
also prepared to establish the spontaneous release of label giving the
30 assay background. Target ⁵¹Cr labelled JY cells are added to all wells in
10µl. The same number of JY cells are also added to wells containing 2%
Triton x100 in water to establish the 100% release value. Target cells and
antibody are incubated together and, after 1 hour at room temperature,
25µl serum as a source of complement is added to all wells (except the
35 100%) for a further 1 hour at room temperature. 100µl of EDTA saline at
4°C is then added to stop any further cell killing, the microtitre plates are

centrifuged at 200g to pellet the intact cells and 100 μ l supernatant is removed and counted in a gamma counter.

Percent cell lysis is calculated by subtracting the background from all values and then expressing them as a percentage of the adjusted maximum release. Replicates vary by less than 5%. Percent cell lysis is then plotted against antibody dilution.

Results

10 The ability of L243 to fix human complement was not affected by all the changes made in the N-terminal region of the C_H2 domain, residues 233 to 237 (Figure 11). Wild type IgG1 mediated potent killing with 600ng/ml giving half maximum cell killing (64% maximum). IgG2 and IgG4 caused no cell killing even at 20 μ g/ml. The Gly to Ala at 237 gave an intermediate level killing (20% maximum killing at 2 μ g/ml). Exchanging the whole lower hinge region with the sequence found in human IgG2 failed to cause lysis even at 20 μ g/ml. Changes at 235 in IgG1 had unexpectedly profound effects on human complement fixation. Changing the Leu 235 to Glu abolished complement lysis (no killing at 20 μ g/ml). Ala at 235 permitted low levels of killing. In contrast, a change in the previously described C1q binding motif [Duncan A R and Winter G (1998), Nature, 332, 21.], from Lys to Ala at 320 effected no change from the IgG1 wild type killing (70% maximum cell killing and half the cells dead with 600ng/ml).

25 **DIRECT BINDING OF C1q**

Measurement of the direct binding of human C1q to different engineered variants of L243 was established to confirm that complement mediated cytotoxicity was due to activation of the classical pathway.

30 Purified human C1q (Sigma UK) was directly labelled with fluorescein isothiocyanate (FITC Sigma) using conventional methods. All dilutions, manipulations and incubations were done in Phosphate Buffered Saline (Gibco UK) containing 0.1% Sodium Azide (BDH UK) and 5% Foetal Calf Serum (Sigma UK). 5x10⁴ indicator cells (JY B lymphoblastoid cell line bearing high levels of HLA-DR) were coated with the different engineered antibodies by incubating at saturating concentrations for 1 hour at 4°C in

- RB polystyrene tubes (2052 12x75mm Falcon UK). After washing, serial dilutions of FITC labelled C1q in 100µl were added and were incubated together for a further 30 min at 4°C. After washing, binding of C1q was revealed using a Fluorescence Activated Cell Scanner (FACS Becton Dickinson). After appropriate analysis, median fluorescence intensity is plotted against C1q concentration.

Results

- Direct binding of human C1q to the L243 human isotype series confirmed the results with complement mediated cytotoxicity (Figure 12). Labelled human C1q bound well to wild type IgG1, when bound to JY cells, and bound poorly to IgG4. Equilibrium dissociation constants were determined essentially as described by Krause *et al.* [Behring Inst. Mitt. 87 56 (1990)] and were 1.2×10^{-7} M and 1.5×10^{-8} M for IgG4 and IgG1 respectively.
These values compare favourably with those obtained for the mouse antibodies IgG1 and IgG2a which have similar functions [Leatherbarrow and Dwek (1984), Molec. Immunol. 21, 321]. The Leu 235 to Glu change in IgG1 reduced the binding of C1q to the same level as IgG4. In contrast, a change in the previously described C1q binding motif [Duncan A R and Winter G (1988) Nature 332, 21], from Lys to Ala at 320 had no effect on C1q binding. The Leu 235 to Glu change in IgG4 did not alter wild type binding.

Rabbit and Guinea Pig complement

- The G1[L235E] and G1[L235A] modifications behaved differently when rabbit or guinea pig serum was used as a source of complement instead of human. With rabbit C' they caused the same level of lysis as the wild type G1. With guinea pig they caused 40% and 49% plateau level killing, respectively, compared with 80% killing by the IgG1 wild type. The 235 change only affects human complement binding indicating that rabbit and guinea pig complement interact differently with human IgG1 (see Figures 13-15).

ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY.

- The ability of the engineered variants of L243 to bind to Fc_gRIII was assessed using antibody dependent cell mediated cytotoxicity (ADCC).

The principle of the experiment is that antibodies will mediate lysis of target cells bearing their cognate antigen if the Fc of the antibody is able to interact with Fc receptor bearing effector cells capable of cytotoxicity. The 5 critical interaction is between antibody Fc and cellular Fc receptors.

- Effector cells are prepared fresh for each experiment. Human venous blood is drawn into endotoxin free tubes containing heparin. Peripheral blood mononuclear cells (PBMC) are prepared by density gradient 10 centrifugation according to the manufacturers instructions (Pharmacia). PBMC are adjusted to 1×10^7 cells/ml in RPMI 1640 medium (Gibco UK) containing 2mM Glutamine (Gibco UK) and 10% foetal calf serum (Sigma UK), in which all manipulations, dilutions and incubations are done.
- 15 Target cells (JY B lymphoblastoid cell line bearing high levels of HLA-DR) are labelled with 1mCi Na⁵¹Cr for 1 hour at room temperature, agitated every 15 min. The cells are then washed three times, to remove free radiolabel, and resuspended at 2×10^6 /ml. Serial antibody dilutions are prepared in duplicate in sterile U-bottom 96 well microtitre plates (Falcon 20 UK) in 25µl. Control wells containing medium only are also prepared to establish the spontaneous release of label giving the assay background. Target ⁵¹Cr labelled JY cells are added to all wells in 10µl. The same number of JY cells are also added to wells containing 2% Triton x100 in water to establish the 100% release value. Target cells and antibody are 25 incubated together and, after 30min at room temperature, 25µl effector cells are added to all wells (except the 100%) for a further 4 hours at 37°C. 100µl of EDTA saline at 4°C is then added to stop any further cell killing, the microtitre plates are centrifuged at 200g to pellet the intact cells and 100µl supernatant is removed and counted in a gamma counter.
- 30 Percent cell lysis is calculated by subtracting the background from all values and then expressing them as a percentage of the adjusted maximum release. Replicates vary by less than 5%. Percent cell lysis is then plotted against antibody dilution.
- 35

Results

Not all the changes made in the N-terminal region of the CH2 domain, residues 233 to 237, affected FcRIII mediated function (Figure 16 and Tables 5 and 7). L243 IgG2 was unable to mediate peripheral blood

- 5 mononuclear cell cytotoxicity (ADCC) of HLA-DR positive JY lymphoblastoid cells at concentrations up to 100 μ g/ml. IgG4 caused a low level of ADCC (20% maximum killing at 1 γ /ml) which could be abrogated by the Leu 235 to Glu change. Wild type IgG1 was a potent mediator of cell killing giving 50% cell death at 5ng/ml antibody. Gly to Ala at 237 reduced the
10 IgG1 wild type killing to the level seen with IgG4. Exchanging the whole lower hinge region with the sequence found in human IgG2 gave intermediate levels of killing with 500ng/ml needed for 50% cell death. In contrast, changes at 235 in IgG1 had minimal effect on ADCC.
- 15 Changing the Leu 235 to Ala gave levels of killing comparable with the G1 wild type (9ng/ml for 50% cell death) and changing the Leu 235 to Glu reduced ADCC a little (40ng/ml for 50% cell death). A change in the previously described C1q binding motif, from Lys to Ala at 320 had no effect on the ability of IgG1 to mediate ADCC.

20

IMMUNE FUNCTION

- Ex vivo T cell function experiments were performed where an interaction between MHC-II and the T cell receptor was an obligatory requirement for T cell activation. The L243 isotype series was tested in mixed lymphocyte reactions, which measures both naive and memory T cell activation, and recall responses to tetanus toxoid which only measures a memory T cell response.

Mixed Lymphocyte Reaction.

- 30 The immunosuppressive potency of engineered variants of L243 was assessed using a mixed lymphocyte reaction.

- The principle of the experiment is that when leucocytes from one individual are mixed with those of another which express different HLA alleles, they
35 will recognise each other as foreign and will become activated. This activation is dependent, primarily, on interactions between the CD3/TcR

complex on T cells and the MHC-II molecule on antigen presenting cells. Antibodies that bind to MHC-II are known to inhibit this reaction.

- Leucocytes are prepared fresh for each experiment. Human venous blood
5 from two individuals is drawn into endotoxin free tubes containing heparin. Peripheral blood mononuclear cells (PBMC) are prepared by density gradient centrifugation according to the manufacturers instructions (Pharmacia). PBMC are adjusted to 2×10^6 cells/ml in RPMI 1640 medium (Gibco UK) containing 2mM Glutamine (Gibco UK), 100 μ M/100 μ g/ml
10 Penicillin/ Streptomycin (Gibco) and 10% foetal calf serum (Sigma UK), in which all manipulations, dilutions and incubations are done. PBMC from one individual are irradiated with 3000 rads. These cells will be stimulate a response from the other individual.
- 15 Serial antibody dilutions are prepared in triplicate in sterile U-bottom 96 well microtitre plates (Falcon UK) in 100 μ l. Control wells containing medium only and optimal Cyclosporin (Sandimmun®, Sandoz) levels (100nM) are also prepared to establish the maximum response and maximum inhibition, respectively. Equal numbers of irradiated stimulators
20 and responders are mixed together and 100 μ l are added to each well. Wells of stimulator alone and responders alone are also set up as controls. The experiment is incubated at 37°C in 100% humidity and 5%CO₂ for 5 days. Response is measured by assessing proliferation during the last 18 hours of culture by incubation with 1 μ Ci/well ³H-Thymidine (Amersham
25 UK), harvesting on to glass filter mattes and counting using a beta counter.

Results are plotted as CPM against antibody concentration. Replicates vary by less than 10%.

- 30 **T cell Recall Response to Tetanus Toxoid**
The ability of the engineered variants of L243 to suppress a secondary response was assessed using a recall response to Tetanus Toxoid.
35 The principle of the experiment is that T lymphocytes from an individual previously immunised with Tetanus Toxoid (TT) will respond to TT when re-exposed ex vivo. This activation is dependent on the interaction

between the CD3/TcR complex on T cells and the MHC-II molecule on cells which process and present the antigen. Antibodies that bind to MHC-II are known to inhibit this reaction.

- 5 Lymphocytes are prepared fresh for each experiment. Human venous blood is drawn into endotoxin free tubes containing heparin. Peripheral blood mononuclear cells (PBMC) are prepared by density gradient centrifugation according to the manufacturers instructions (Pharmacia). PBMC are adjusted to 2×10^6 cells/ml in RPMI 1640 medium (Gibco UK)
- 10 containing 2mM Glutamine (Gibco UK), 100 μ /ml/100 μ g/ml Penicillin/Streptomycin (Gibco) and 10% foetal calf serum (Sigma UK), in which all manipulations, dilutions and incubations are done.

- Serial antibody dilutions are prepared in triplicate in sterile U-bottom 96-well microtitre plates (Falcon UK) in 100 μ l. 50 μ l containing an optimal concentration of TT, previously determined by experimentation, is added to all wells. Control wells containing medium only or Cyclosporin (Sandimmun, Sandoz) (100nM) are also prepared to establish the maximum response and maximum inhibition, respectively. 50 μ l PBMC are then added to each well. The experiment is incubated at 37°C in 100% humidity and 5%CO₂ for 7 days. Response is measured by assessing proliferation during the last 18 hours of culture by incubation with 1 μ Ci/well ³H-Thymidine, harvesting on to glass filter mattes and counting using a beta counter.

- 25
- Results are plotted as CPM against antibody concentration. Replicates vary by less than 10%.

Results (Figures 17-21)

- 30 There were no significant or qualitative differences between the effects of the L243 human isotype series between the MLR and TT response. Maximal inhibition was achieved with G1, G1[L235E] and G1[L235A]. Approximately two orders of magnitude more of G2, G4 and G1[G237A] was required to give similar levels of inhibition. The G1/G2 L hinge exchange mutant was intermediate in immuno-suppressor potency. There was no correlation between complement fixation or FcRI binding and
- 35

immuno-suppression, G1 binding well to FcRI and fixing complement and G1[L235E] doing neither, but both giving good immunosuppression. But, there was good correlation with FcRIII binding. Human G1 and G1[L235E] interact with FcRIII and give good immunosuppression. The G1/G2 L 5 hinge is intermediate in FcRIII binding and immuno-suppression. In contrast, the G237A mutation in human G1, in agreement with published observations, reduces FcRIII binding. This antibody gave poor immunosuppression. (Table 5). Table 6 shows a number of L243 isotype mutants.

10

Conclusion

We have found that amino acid residues necessary for C1q and FcR binding of human IgG1 are located in the N-terminal region of the CH2 domain, residues 231 to 238, using a matched set of engineered 15 antibodies based on the anti-HLA DR antibody L243. Changing the leucine 235 in the CH2 region of IgG3 and IgG4 to glutamic acid was already known to abolish FcRI binding, we have confirmed this for IgG1 and also found a concomitant abolition of human complement fixation with retention of FcRIII mediated function. Changing the glycine at 237 to alanine of 20 IgG1 also abolished FcRI binding and reduced complement fixation and FcRIII mediated function. Exchanging the whole region 233 to 236, with the sequence found in human IgG2 abolished FcRI binding and complement fixation and reduced FcRIII mediated function of IgG1. In contrast, a change in the previously described C1q binding motif, from 25 lysine at 320 to alanine had no effect on IgG1-mediated complement fixation.

The effect of these changes in IgG1 on FcRI binding are similar to published observations using IgG3 and IgG4 [Lund J *et al* J. Immunol. 30 1991. 147, 265; and Alegre M-L *et al* J. Immunol. 1992. 148, 3461] with changes at 235 and 237 in the lower hinge/N-terminal CH2 region markedly reducing FcRI binding. The similarities between these three isotypes strongly suggests that they interact with FcRI in a similar way.

35 We have found residues necessary for FcRIII binding of human IgG1 within the lower hinge/N-terminal end of the CH2 region. Modification at 237 and

exchanging the lower hinge for IgG2 residues caused low and intermediate levels, respectively, of FcRIII mediated killing. These effects are similar to those reported by Sarmay *et al* [Molec. Immunol. 1992. 29, 633] for human IgG3. In contrast to Sarmay *et al* using IgG3, our changes at residue 235
5 of IgG1 had little effect on FcRIII binding.

Greenwood *et al* [Eur. J. Immunol. 1993. 23, 1098], using inter and intra domain switch variants between IgG1 and IgG4, identify residues in IgG1 necessary for FcRIII binding in the C-terminal half of the CH2 domain
10 beyond 292. This indicates that the residues we have identified within the lower hinge/N-terminal end of the CH2 region are necessary but not sufficient for FcRIII effector function mediated through binding of human IgG1.

15 IgG1 variants with changes at 235 failed to mediate lysis with human complement and did not bind purified human C1q. We also found that an IgG1 molecule containing a change at 320 gave complement mediated killing equivalent to the IgG1 wild type. Residues, Glu 318, Lys 320 and Lys 322 were identified by protein engineering studies as necessary in
20 mouse IgG2b for C1q binding [Duncan, A R and Winter G, Nature, 1988. 322, 21]. The same study also demonstrated that the 235 change in mouse IgG2b left unchanged its affinity for human C1q [Duncan, A R and Winter G, Nature, 1988. 322, 21]. The apparent contradiction between these observations is probably due to differences in C1q contacts between
25 human IgG1 and mouse IgG2b.

We found that most changes in the lower hinge/N-terminal end of the CH2 domain affect C1q binding. The G1/G2 lower hinge exchange abolished complement fixation and the change at 237 also reduces it significantly. In
30 contrast, Greenwood *et al* [Eur. J. Immunol. 1993. 23, 1098], found residues necessary for human complement fixation in the C-terminal half of the CH2 domain. Tao *et al* [J. exp. Med. 1993. 178, 661] also identify the C-terminal half of the CH2 domain as necessary for complement fixation. They are able to separate C1q binding from complement mediated lysis.
35 IgG1 with a Pro to Ser change at 331, in the C-terminal half of the CH2 domain, is able to bind human C1q as well as the wild type but is unable to

activate complement. This predicts that the amino acids that we have identified within the lower hinge/N-terminal end of the CH2 region are necessary for C1q binding and that the C-terminal residues are necessary for the binding and activation of the antibody dependent complement cascade beyond C1q.

TABLE 5

5

Summary of L243 Isotype Series

	L243	RI	RIII	C1q	MLR	TT
10						
G2	-	-	-	-	++	++
G4	+	-	-	-	++	++
15						
G1	+++	+++	+++	+++	++++	++++
G1L235E	-	++±	±	---	+++	+++
20						
G1L235A	+	++	+	+++	++++	++++
G1G237A	+	+	+	++	++	++

TABLE 6

5

Human Isotype Mutants

<u>Gene</u>	<u>Residue</u>	<u>From</u>	<u>To</u>	<u>NAME</u>
G1	235	L	E	G1[L235E]
G1	235	L	A	G1[L235A]
G1	237	G	A	G1[G237A]
G1	320	K	A	G1[K320A]
G4	235	L	E	G4[L235E]
G1	231-238	APELLGGP	AP-PVAGP	G1/G2L-hinge

TABLE 7

5

Summary of L243 Isotype Series

<u>L243</u>	<u>R_I^a</u>	<u>R_{III}^b</u>	<u>Complement^c</u>
G2	>10	>100000	>20/0
G4	1.2	10000ex	>20/0
G4[L235E]	>10	>100000	>20/0
G1	0.13	5	0.6/65
G1/G2Lh	>10	500	>20/0
G1[L235E]	>10	40	>20/0
G1[L235A]	5.0	9	>20/0
G1[G237A]	>10	10000ex	2.0/20
G1[K320A]	0.1	10	0.6/70

- 10 a) mg/ml antibody necessary for 50% inhibition of binding of FITC-labelled mouse IgG2a antibody to U937 cells.
- b) ng/ml antibody necessary for half maximal cell killing in ADCC. (ex) extrapolated value.
- 15 c) mg/ml antibody necessary for half maximal cell killing by human complement and percent plateau cell killing.

EXAMPLE 3

L243 is a mouse monoclonal antibody raised against human MHC Class II. The nucleotide and amino acid sequences of L243 VL and VH are shown in Figures 5 and 3 respectively. The following examples describe the 5 humanisation of the L243 antibody (CDR grafting).

CDR grafting of L243 light chain

Alignment of the framework regions of L243 light chain with those of the four human light chain subgroups [Kabat, E.A., Wu, T.T., Perry, H.M., 10 Gottesman, K.S. and Foeller, C. 1991, Sequences of Proteins of Immunological Interest, Fifth Edition] revealed that L243 was most homologous to antibodies in human light chain subgroup 1. Consequently, for constructing the CDR grafted light chain, the framework regions chosen corresponded to those of the human Group 1 consensus sequence. A 15 comparison of the amino acid sequences of the framework regions of L243 and the consensus human group 1 light chains is given below and shows that there are 21 differences (underlined) between the two sequences.

Analysis of the contribution that any of these framework differences might 20 have on antigen binding (see published International patent application No. WO91/09967) identified 4 residues for investigation; these are at positions 45,49,70 and 71. Based on this analysis, two versions of the CDR grafted light chain were constructed. In the first of these, L243-gL1, residues 25 45,49,70 and 71 are derived from the L243 light chain while in the second, L243-gL2, all residues are human consensus.

Light chain Comparisons

Hu group 1 consensus : DIQMTQSPSSLSASVUGDRUTITC

30 L243 : DIQMTQSPASLSSUGETUTITC

4 4
5 9

Hu Group 1 consensus : WYQQKPGKAPKLLIY

35 L243 : WYRQKQGKSPQLLUF

77

01

Hu Group 1 consensus : GUPSRFSGSGSGTDFLTISSLQPEDFATYYC

5 L243 : GUPSRFSGSGSGTOYSLKINSLQSEDFGDYYC

Hu Group 1 consensus : FGQGTKVEIKR

L243 : FGGTNLEIKR

10

Construction of CDR grafted light chain L243-gL1

The construction of L243-gL1 is given below in detail. The following oligonucleotides were used in the Polymerase Chain Reactions (PCR) to introduce changes into the framework regions of the chimeric light chain:

15

R5043 : 5'GTAGGGAGACCGGGTCACCATCACATGTCGAGCA3'

R5044 : 5'CTGAGGGAGCTTTCTGGTTCTGCTGATACCATGCTAAA3'

R5045 : 5'AAACCCAGGGAAAAAGCTCCTCAGCTCCTGATCTTGCTGCATC3'

R5046 : 5'CTTCTGGCTGCAGGCTGGAGATAGTTAGGGTATACTGTGTGCC3'

20

R5047 : 5'CTTCAGCCTGCAGCCAGAAGATTGCTACTTATTACTGTCA3'

R5048 : 5'GGGCCGCTACCGTACGTTTAGTTCCACTTGGTGCCTGACCGAA3'

Three reactions, each of 20 µl, were set up each containing 10 mM Tris-

HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each

25

deoxyribonucleoside triphosphate, 0.1 µg chimeric L243 light chain DNA,

6 pmoles of R5043/R5044 or R5045/R5046 or R5047/R5048 and 0.25

units Taq polymerase. Reactions were cycled through 94°C for 1 minute,

55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction

30

was analysed by electrophoresis on an agarose gel and the PCR

fragments excised from the gel and recovered using a Mermaid Kit.

Aliquots of these were then subjected to a second round of PCR. The

reaction, 100 µl, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM

KCl, 0.01% w/v gelatin, 1/10 of each of the three PCR fragments from the

35

first set of reactions, 30 pmoles of R5043 and R5048 and 2.5 units Taq

polymerase. Reaction temperatures were as above. After the PCR, the

mixture was extracted with phenol / chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SpII. The resulting product was finally electrophoresed
5 on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 1) that had previously been digested with the same enzymes.

The ligation mixture was used to transform E. coli LM1035 and resulting
10 colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of L243-gL1 is shown in Figure 22.

Construction of CDR grafted light chain L243-gL2

15 L243-gL2 was constructed from L243-gL1 using PCR. The following oligonucleotides were used to introduce the amino acid changes :

R1053 : 5'GCTGACAGACTRACAGACTGTTCC3'

R5350 :

20 5'TCTAGATGGCACACCATCTGCTAAGTTGATGCAGCATAGATCAGGAGCTTAGGA
GC3'

R5349 :

5'GCAGATGGTGTGCCATCTAGATTCAAGTGGCAGTGGATCAGGCACAGACTTACCC
TAAC3'

25 R684 : 5'TTCRACTGCTCATCAGAT3'

Two reactions, each 20 µl, were set up each containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 0.1 µg L243-gL1, 6 pmoles of R1053/
30 R5350 or R5349/R684 and 0.25 units Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragments excised from the gel and recovered using a Mermaid Kit.

Aliquots of these were then subjected to a second round of PCR. The reaction, 100 µl, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 1/5 of each of the PCR fragments from the first set of reactions, 30 pmoles of R1053 and R684 and 2.5 units Taq polymerase.

- 5 Reaction temperatures were as above. After the PCR, the mixture was extracted with phenol / chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SphI. The resulting product was finally electrophoresed
10 on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 1) that had previously been digested with the same enzymes.

15 The ligation mixture was used to transform E. coli LM1035 and resulting colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of L243-gL2 is shown in Figure 23.

CDR grafting of L243 heavy chain

- 20 CDR grafting of L243 heavy chain was accomplished using the same strategy as described for the light chain. L243 heavy chain was found to be most homologous to human heavy chains belonging to subgroup 1 and therefore the consensus sequence of the human subgroup 1 frameworks was chosen to accept the L243 heavy chain CDRs.
25
A comparison of the framework regions of the two structures is shown below where it can be seen that L243 differs from the human consensus at 28 positions (underlined). After analysis of the contribution that any of these might make to antigen binding, only residues 27,67,69,71,72 and 75
30 were retained in the CDR grafted heavy chain, L243-gH.

Heavy chain comparisons

2

7

- 35 Hu Group 1 consensus : QUQLVQSGRAEKKPGASUKUSCKASGYTFT
L243 : QIQLVQSGPELKKPGGETVKISCKASGFTFT

	Hu Group 1 consensus :	WURQAPGQGLEWMG
	L243	: W <u>U</u> K <u>Q</u> AP <u>G</u> K <u>G</u> L <u>K</u> WMG
		6 6 77 7
5		7 9 12 5
	Hu Group 1 consensus :	RUTITADTSTSTAYMELSSLRSEDTAVYYCAR
	L243	: R <u>F</u> A <u>F</u> S <u>L</u> E <u>T</u> S <u>A</u> S <u>T</u> A <u>Y</u> <u>L</u> O <u>I</u> N <u>N</u> L <u>K</u> N <u>E</u> D <u>T</u> A <u>K</u> Y <u>F</u> C <u>A</u> R
	Hu Group 1 consensus :	WGQQGTLUTUSS
10	L243	: WGQQ <u>G</u> T <u>T</u> L <u>T</u> USS

Construction of CDR grafted heavy chain, L243 gH

L243gH was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following oligonucleotides, 15 were used in the PCR:

	R3004 :	5'GGGGGGGARGCTTGCCGCCACCATGG3'
	R3005 :	5'CCCCCCC <u>GGG</u> CC <u>TTT</u> G <u>T</u> GA <u>AG</u> C <u>AG</u> 3'
20	R4902 :	5'GAC <u>AC</u> C <u>AGG</u> <u>AG</u> T <u>G</u> C <u>ACT</u> <u>C</u> T <u>C</u> <u>AGG</u> T <u>G</u> C <u>AG</u> C <u>T</u> <u>GG</u> T <u>G</u> C <u>AG</u> T <u>C</u> <u>GG</u> <u>AG</u> C
		AG <u>GGG</u> T <u>G</u> A <u>AG</u> A <u>AG</u> C <u>CT</u> <u>GG</u> <u>G</u> C <u>AT</u> <u>CT</u> G3'
25	R4903 :	5'ACATT <u>CAC</u> <u>AA</u> <u>TT</u> <u>AC</u> <u>GG</u> <u>AA</u> <u>AT</u> <u>G</u> <u>AA</u> <u>TT</u> <u>GGG</u> T <u>G</u> <u>AG</u> <u>AC</u> <u>GG</u> <u>CA</u> <u>CT</u> <u>GG</u> <u>A</u>
		C <u>AGG</u> <u>G</u> <u>ACT</u> <u>C</u> <u>G</u> <u>AG</u> <u>T</u> <u>GG</u> <u>A</u> 3'
	R4904 :	5'CCT <u>ACG</u> <u>T</u> <u>AC</u> <u>GG</u> <u>C</u> <u>AG</u> <u>AC</u> <u>GR</u> <u>CT</u> <u>T</u> <u>CA</u> <u>AG</u> <u>GG</u> <u>AA</u> <u>AG</u> <u>AT</u> <u>T</u> <u>CA</u> <u>C</u> <u>ATT</u> <u>CA</u> <u>CA</u> <u>CT</u> <u>G</u>
		<u>G</u> <u>AG</u> <u>AC</u> <u>AT</u> <u>CT</u> <u>G</u> <u>C</u> <u>AT</u> <u>T</u> <u>AC</u> <u>AG</u> <u>C</u> <u>AT</u> <u>AC</u> <u>AT</u> 3'
30	R4905 :	5'C <u>AG</u> <u>C</u> <u>AG</u> <u>T</u> <u>G</u> <u>T</u> <u>A</u> <u>T</u> <u>C</u> <u>T</u> <u>G</u> <u>C</u> <u>A</u> <u>AG</u> <u>AG</u> <u>AC</u> <u>AT</u> <u>T</u> <u>AC</u> <u>AG</u> <u>C</u> <u>AG</u> <u>T</u> <u>GG</u> <u>T</u> <u>AC</u> <u>CT</u> <u>A</u>
		<u>C</u> <u>AG</u> <u>G</u> <u>AT</u> <u>T</u> <u>C</u> <u>G</u> <u>ACT</u> <u>A</u> <u>T</u> <u>GGG</u> <u>GG</u> <u>A</u> <u>C</u> <u>AGG</u> <u>GA</u> 3'
	R4897 :	5'T <u>G</u> <u>A</u> <u>G</u> <u>AG</u> <u>T</u> <u>G</u> <u>C</u> <u>AC</u> <u>T</u> <u>C</u> <u>C</u> <u>T</u> <u>G</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>A</u> <u>C</u> <u>AG</u> <u>G</u> <u>A</u> <u>AG</u> <u>A</u> <u>AC</u> <u>GG</u> <u>A</u> <u>AC</u> <u>CC</u>
		<u>C</u> <u>AG</u> <u>A</u> <u>CC</u> <u>AC</u> <u>T</u> <u>CC</u> <u>AT</u> <u>GG</u> <u>T</u> <u>GG</u> <u>CG</u> <u>GG</u> <u>C</u> <u>A</u> <u>AG</u> <u>C</u> <u>T</u> <u>CC</u> <u>CC</u> <u>CC</u> 3'
35	R4898 :	5'CAT <u>CC</u> <u>GT</u> <u>A</u> <u>TT</u> <u>T</u> <u>G</u> <u>AA</u> <u>T</u> <u>G</u> <u>AT</u> <u>T</u> <u>C</u> <u>C</u> <u>AG</u> <u>AT</u> <u>G</u> <u>C</u> <u>CT</u> <u>T</u> <u>AC</u> <u>A</u> <u>AG</u> <u>A</u> <u>AC</u> <u>C</u>
		<u>CT</u> <u>T</u> <u>CA</u> <u>C</u> <u>AG</u> <u>AT</u> <u>G</u> <u>C</u> <u>TC</u> <u>C</u> <u>AG</u> <u>G</u> <u>CT</u> <u>T</u> <u>CT</u> <u>TC</u> 3'
	R4899 :	5'G <u>R</u> <u>A</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>A</u> <u>GG</u> <u>C</u> <u>T</u> <u>C</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>CC</u> <u>A</u>
40		<u>T</u> <u>CC</u> <u>C</u> <u>AT</u> <u>CC</u> <u>AC</u> <u>T</u> <u>C</u> <u>G</u> <u>AG</u> <u>T</u> <u>CC</u> <u>CT</u> <u>G</u> <u>T</u> <u>CC</u> <u>AG</u> 3'

R4900 : 5' TTGCACAGTAGTACACTGCTGTGTCCTCAGATCTCAGAGAAGACA
GCTCCATGTATGCTGTAAGATGCAGAT3'

5 R4901 : 5' CCCCCCGGGCCCTTGATGAAAGCAGRAAGACACTGTCACCRAGTGT
CCCTGTCCCCAGTAGTCGAA3'

The assembly reaction, 50 µl, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 1 pmole of each of R4897 - R4905, 10 pmoles of each of R3004 and R3005 and 2.5 units Taq polymerase. Reactions were cycled through 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute. After 10 30 cycles, the reaction was extracted with phenol/chloroform (1/1), then with chloroform and precipitated with ethanol. After centrifugation, the DNA 15 was dissolved in the appropriate restriction buffer and digested with HindIII and Apal. The resulting fragment was isolated from an agarose gel and ligated into pMR14 (Figure 2) that had previously been digested with the same enzymes. pMR14 contains the human gamma 4 heavy chain constant region and so the heavy chain expressed from this vector will be a 20 gamma 4 isotype. The ligation mixture was used to transform E. coli LM1035 and resulting bacterial colonies screened by restriction digest and nucleotide sequence analysis. In this way, a plasmid containing the correct sequence for L243gH was identified (Figure 24).

25 Construction of Gamma 1 versions of chimeric and CDR grafted L243 heavy chain

Human Gamma 1 versions of L243 heavy chains were constructed by transferring the variable regions of both the murine and the CDR grafted 30 heavy chains as HindIII to Apal fragments into the vector pGamma1 (Figure 6). This vector contains the human Gamma 1 heavy chain constant region.

Evaluation of activities of CDR grafted genes

The activities of the CDR grafted genes were evaluated by expressing 35 them in mammalian cells and purifying and quantitating the newly synthesised antibodies. The methodology for this is described next,

followed by a description of the biochemical and cell based assays used for the biological characterisation of the antibodies.

a) Gene Expression in CHO cells

- 5 Chimeric and CDR grafted L243 was produced for biological evaluation by transient expression of heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation as described above for production of chimeric L243.
- 10 Antibody concentration was determined using a human Ig ELISA (see below).

b) ELISA

- For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a F(ab)2 fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson Immuno-research, code 109-006-098) at 5 µg/ml in coating buffer (15mM sodium carbonate, 35mM sodium hydrogen carbonate, pH6.9). Uncoated antibody was removed by washing 5 times with distilled water. Samples and purified standards to be quantitated were diluted to approximately 1 µg/ml in conjugate buffer (0.1M Tris-HCl pH7.0, 0.1M NaCl, 0.2% v/v Tween 20, 0.2% w/v Hammersten casein). The samples were titrated in the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the plates incubated at room temperature for 1 hr with shaking. After the first incubation step the plates were washed 10 times with distilled water and then incubated for 1 hr as before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate buffer. The plate was washed again and substrate solution (0.1 ml) added to each well. Substrate solution contained 150µl N,N,N,N-tetramethylbenzidine (10 mg/ml in DMSO), 150µl hydrogen peroxide (30% solution) in 10 ml 0.1M sodium acetate/sodium citrate, pH6.0. The plate was developed for 5 -10 minutes until the absorbance at 630nm was approximately 1.0 for the top standard. Absorbance at 630nm was measured using a plate reader and the concentration of the sample determined by comparing the titration curves with those of the standard.

c) Competition Assay

The principle of this assay is that if the antigen binding region has been correctly transferred from the murine to human frameworks, then the CDR grafted antibody will compete equally well with a labelled chimeric antibody

- 5 for binding to human MHC Class II. Any changes in the antigen binding potency will be revealed in this system.

Chimeric L243 was labelled with fluorescein (FITC) using the method of

Wood et al [Wood,T., Thompson, S and Goldstein, G 1965, J. Immunol 95,

- 10 225-229 and used in the competition assay described above.

Figure 25 compares the ability of combinations of L243 heavy and light chains to compete with FITC-labelled chimeric L243 for binding to JY cells.

All combinations were effective competitors although none of those

- 15 containing CDR grafted heavy or light chains were as effective as the chimeric antibody itself. Thus, the combinations cH/gL1, gH/cL and gH/gL1 were 89%, 78% and 64% respectively, as effective as chimeric L243 in this assay.

20 d) Determination of Affinity constants by Scatchard Analysis

L243 antibodies were titrated from 10 μ g/ml in PBS, 5% fetal calf serum,

0.1% sodium azide in 1.5-fold dilutions (150 μ l each) before incubation with

5 \times 10⁴ JY cells per titration point for 1 hour on ice. The cells were previously counted, washed and resuspended in the same medium as the

25 samples. After incubation, the cells were washed with 5ml of the above medium, spun down and the supernatant discarded. Bound antibody was revealed by addition of 100 μ l of a 1/100 dilution of FITC conjugated anti-

human Fc monoclonal (The Binding Site; code MF001). The cells were then incubated for 1 hour on ice and then the excess FITC conjugate

30 removed by washing as before. Cells were dispersed in 250 μ l of the same buffer and the median fluorescence intensity per cell was determined in a

FACScan (Becton Dickinson) and calibrated using standard beads (Flow Cytometry standards Corporation). The number of molecules of antibody bound per cell at each antibody concentration was thus established and

35 used to generate Scatchard plots. For the purpose of calculation, it was

assumed that the valency of binding of the FITC conjugate to L243 was 1:1 and that the F/P ratio was 3.36 (as given by the manufacturer).

A Scatchard plot comparing the affinities of chimeric L243 (cH/cL), L243-5 gH/L243-gL1 and L243-gH/L243-gL2 is shown in Figure 26. Chimeric L243 was found to have an apparent Kd of 4.1 nM while the CDR grafted antibodies containing gL1 and gL2 light chains had apparent Kd of 6.4nM and 9.6nM respectively. The difference in Kd values of the antibodies with the two CDR grafted light chains reflects the contribution made by residues 10 45,49,70 and 71 that had been retained, in L243-gL1, from the parent light chain.

e) Antibody dependent cell mediated cytotoxicity

The ability of chimeric and CDR grafted L243 to mediate antibody 15 dependent cell cytotoxicity (ADCC) was compared as described previously. The principle of the experiment is that antibodies will mediate lysis of target cells bearing their cognate antigen if the Fc of the antibody is able to interact with Fc receptor bearing effector cells capable of cytotoxicity.

20 A comparison of the activities of chimeric (cH/cL) and CDR grafted (gH/gL1) L243 human gamma 1 isotypes in the above assay is shown in Figure 27. Both antibodies were effective mediators of cell lysis with maximal activity being achieved at antibody concentrations of less than 100 ng/ml. There was no significant difference between the activities of the 25 two antibodies.

f) Immune function tests

Ex vivo T cell function experiments were performed where an interaction between MHC-II and the T cell receptor was an obligatory requirement for 30 T cell activation. Chimeric and CDR grafted L243 antibodies were compared in mixed lymphocyte reactions, which measures both naive and memory T cell activation, and in recall responses to tetanus toxoid which only measures a memory T cell response.

1) Mixed Lymphocyte reaction - as described above

The principle of the experiment is that when leucocytes from one individual are mixed with those of another individual which express different HLA alleles, they will recognise each other as foreign and the lymphocytes will

- 5 become activated. This activation is dependent primarily on interactions between the CD3/TcR complex on T cells and the MHC Class II molecule on antigen presenting cells. L243 is known to inhibit this reaction.

When an MLR was carried out to compare the effectiveness of the Gamma

- 10 1 isotypes of chimeric and CDR grafted L243 as inhibitors of T cell activation, no significant differences were observed between the two antibodies (Figure 28). Greater than 90% inhibition of the MLR was observed using 100 ng/ml of either antibody.

15 2) T cell recall response to Tetanus toxoid

The ability of chimeric and CDR grafted L243 to suppress a secondary response was assessed using a recall response to Tetanus toxin. The principle of the experiment is described above.

- 20 The results of an experiment comparing the ability of human gamma 1 isotypes of chimeric and CDR grafted L243 to inhibit the response to TT is shown in Figure 29. Both antibodies were effective inhibitors of the T cell response to TT and produced titration curves that were indistinguishable.

25 **EXAMPLE 4**

The ability of CDR grafted L243 with the alteration at position 235 i.e. L[235E] to mediate antibody dependent cell cytotoxicity (ADCC) was measured essentially as described in the previous examples. The results are shown in Figure 27.

30

Similarly the CDR grafted L243 [L235E] antibody was tested in a mixed lymphocyte reaction and in recall response to tetanus toxoid essentially as described in the previous Examples. The results are provided in Figures 28 and 29.

35

The ability of the CDR-grafted L243 antibody [L235E] to fix human complement was assessed using the technique of antibody dependent complement mediated cytotoxicity as described in the previous Examples. The results are shown in Figure 30.

CLAIMS

1. An altered antibody wherein one or more amino acid residues in the
5 N-terminal region of the C_H2 domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody.
2. An antibody according to Claim 2 which binds to one or more cellular
10 Fc receptors and does not bind significantly to FcR1.
3. An antibody according to Claim 1 or 2 wherein the amino acid residue
which is altered lies within amino acid positions 231 to 239.
- 15 4. An antibody according to any of the preceding claims which is an MHC specific antibody.
5. A method for producing an altered antibody with altered ability to fix
complement as compared to unaltered antibody comprising altering
20 one or more amino acids in the N-terminal region of the C_H2 domain of said antibody altering the ability of said antibody to fix complement as compared with unaltered antibody.
- 25 6. A method of modulating the function of cell surface associated
antigens avoiding complement mediated toxicity comprising administration of an altered antibody wherein one or more amino acid residues in the N-terminal region of the C_H2 domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody and also
30 said antibody.
7. A method according to Claim 6 wherein said altered antibody is able to bind one or more cellular Fc receptors especially FcRIII while binding to FcRI is significantly reduced.

8. A therapeutic, diagnostic or pharmaceutical composition comprising an altered antibody according to any of the preceding claims.
- 5 9. A process for the preparation of a therapeutic, pharmaceutical or diagnostic composition comprising admixing an altered antibody according to any of the preceding claims together with a pharmaceutically acceptable excipient, diluent or carrier.
- 10 10. A method of therapy and diagnosis comprising administering an effective amount of an altered antibody according to any of the preceding claims to a human or animal subject.
- 15 11. A process for producing an altered antibody according to any of the preceding claims comprising:
 - a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain
 - b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain
 - 20 c) transfecting a host cell with both operons and
 - d) culturing the transfected cell line to produce the antibody molecule.
- 25 12. A process according to Claim 11 wherein said DNA sequences encode a humanised antibody.
- 30 13. A process according to Claim 12 wherein said DNA sequences encode a CDR-grafted heavy and/or light chain, or a chimeric antibody
- 35 14. A process according to Claim 11, 12 or 13 wherein at least one of the expression vectors contains a DNA sequence encoding an antibody heavy chain in which one or more amino acid residues in the N-terminal region of the CH₂ domain has been altered from that in the corresponding unaltered antibody.

15. A process according to Claim 11 or 12 wherein the alteration in the N-terminal region of the C_H2 domain is made after the unaltered antibody has been expressed.
5
16. An altered antibody according to any of the preceding claims derived from the anti-MHC antibody L243 (ATCC HB55).
17. An altered antibody according to any of the preceding claims wherein the lower hinge of said antibody has been exchanged with an antibody of different isotype.
10
18. An altered antibody according to Claim 17 wherein an IgG1 lower hinge region has been exchanged with a IgG2 lower hinge region.
19. A method of treating diseases in which antibody therapy leads to undesirable toxicity due to antibody mediated complement fixation comprising administering an altered antibody wherein one or more amino acid residues in the N-terminal region of the C_H2 domain of said antibody are altered characterised in that the ability of said antibody to fix complement is altered as compared to unaltered antibody.
15
- 20

FIG. 1

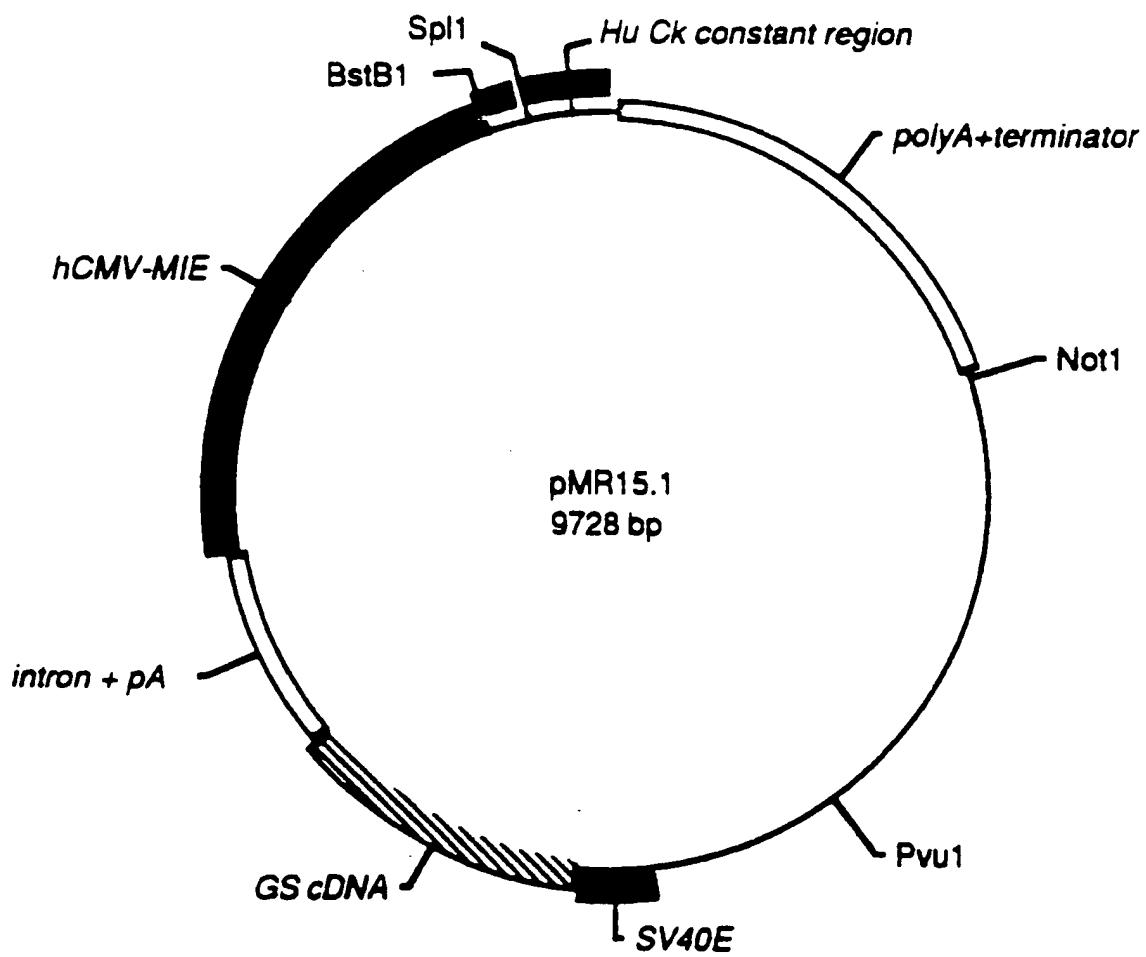
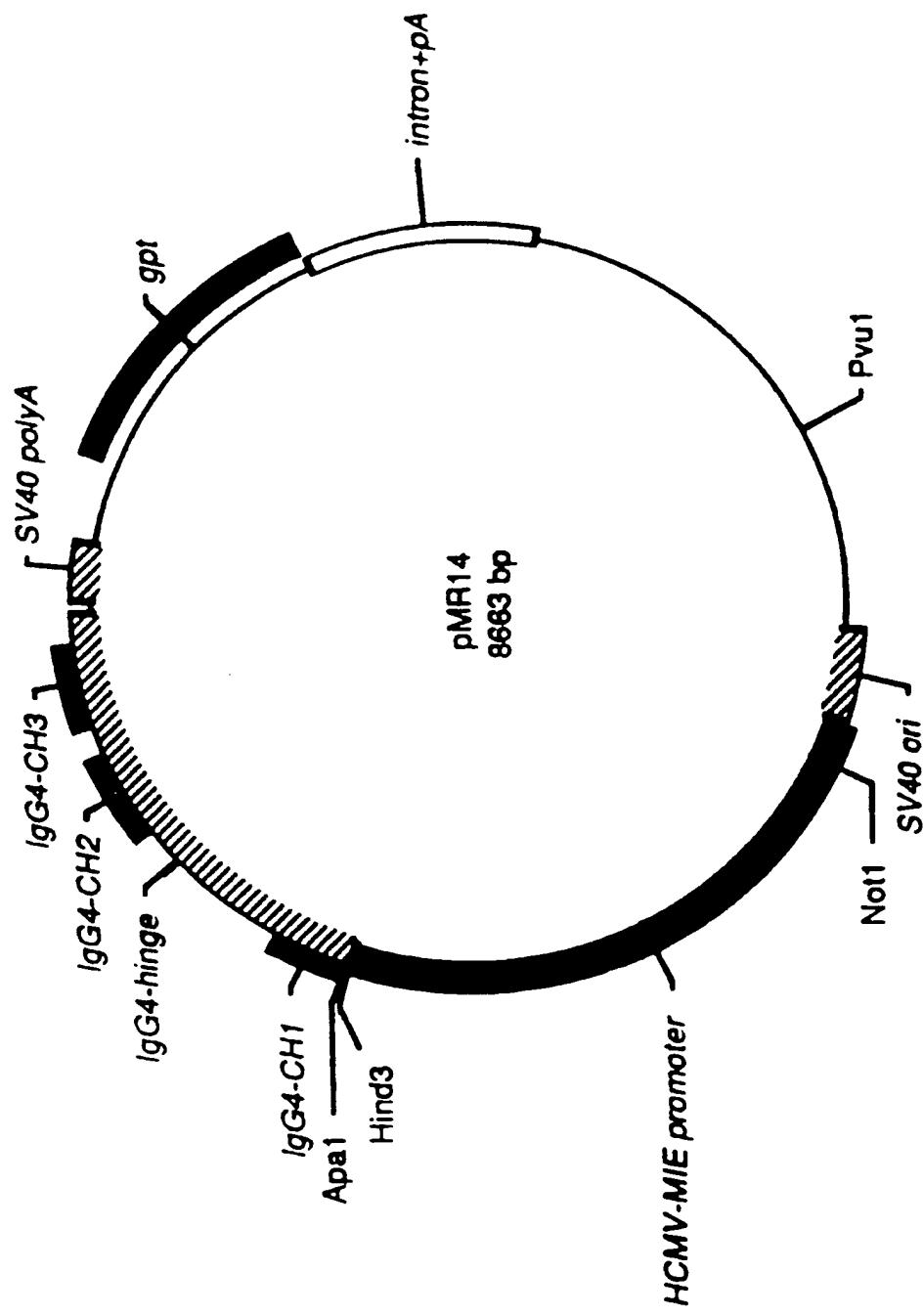


FIG. 2



3 / 30

FIG. 3

AAG CTT GCC GCC ACC ATG GCT TGG GTG TGG AAC TTG CTA TTC CTG ATG
 TTC GAA CGG CGG TGG TAC CGA ACC CAC ACC TTG AAC GAT AAG GAC TAC
 M A W V W N L L F L M>

GCA GCT GCC CAA AGT GCC CAA GCA CAG ATC CAG TTG GTG CAG TCT GGA
 CGT CGA CGG GTT TCA CGG GTT CGT GTC TAG GTC AAC CAC GTC AGA CCT
 A A A Q S A Q A Q I Q L V Q S G>

CCT GAG CTG AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCT
 GGA CTC GAC TTC TTC GGA CCT CTC TGT CAG TTC TAG AGG ACG TTC CGA
 P E L K K P G E T V K I S C K A>

TCT GGG TTT ACC TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT
 AGA CCC AAA TGG AAG TGT TTG ATA CCT TAC TTG ACC CAC TTC GTC CGA
 S G F T F T N Y G M N W V K Q A>

CCA GGA AAG GGT TTA AAG TGG ATG GGC TGG ATA AAC ACC TAC ACT AGA
 GGT CCT TTC CCA AAT TTC ACC TAC CCG ACC TAT TTG TGG ATG TGA TCT
 P G K G L K W M G W I N T Y T R>

GAG CCA ACA TAT GCT GAT GAC TTC AAG GGA CGG TTT GCC TTC TCT TTG
 CTC GGT TGT ATA CGA CTA CTG AAG TTC CCT GCC AAA CGG AAG AGA AAC
 E P T Y A D D F K G R F A F S L>

GAA ACC TCT GCC AGC ACT GCC TAT TTG CAG ATC AAC AAC CTC AAA AAT
 CTT TGG AGA CGG TCG TGA CGG ATA AAC GTC TAG TTG TTG GAG TTT TTA
 E T S A S T A Y L Q I N N L K N>

GAG GAC ACG GCT AAA TAT TTC TGT GCA AGA GAT ATT ACT GCG GTT GTA
 CTC CTG TGC CGA TTT ATA AAG ACA CGT TCT CTA TAA TGA CGC CAA CAT
 E D T A K Y F C A R D I T A V V>

CCT ACG GGT TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACC GTC TCC
 GGA TGC CCA AAA CTG ATG ACC CCG GTT CCG TGG TGA GAG TGG CAG AGG
 P T G F D Y W G Q G T T L T V S>

TCA
 AGT
 S>

FIG. 4

4 / 30

a. TTC GAA GCC GCC ACC ATG TGG GGA TCT GTT TTC CAT TTT TCA ATT GTA
 AAG CTT CGG CGG TGG TAC ACC CCT AGA CAA AAG GTA AAA AGT TAA CAT
 M W G S V F H F S I V>

GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA TCT
 CTA CGG TCT ACA CTG TAG GTC TAC TGA GTC AGA GGT CGG AGG GAT AGA
 D A R C D I Q M T Q S P A S L S>

GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT
 CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA
 V S V G E T V T I T C>

b. TTC GAA GCC GCC ACC ATG AGG TGC TCT GCT GAG TTT CTG GGG TTG CTG
 AAG CTT CGG CGG TGG TAC TCC ACG AGA CGA CTC AAA GAC CCC AAC GAC
 M R C S A E F L G L L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG TCT
 GAC GAC ACC GAA TGT CTA CGG TCT ACA CTG TAG GTC TAC TGA GTC AGA
 L L W L T D A R C D I Q M T Q S>

CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT
 GGT CGG AGG GAT AGA CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA
 P A S L S V S V G E T V T I T C>

c. TTC GAA GCC GCC ACC ATG GGC ATC AAG ATG GAG TCA CAG TTC CAG GTC
 AAG CTT CGG CGG TGG TAC CCG TAG TTC TAC CTC AGT GTC AAG GTC CAG
 M G I K M E S Q F Q V>

TTC ATA TCC ATA CTG CTC TGG TTA TAT GGA GCT GAT GGG AAC ATT GTA
 AAG TAT AGG TAT GAC GAG ACC AAT ATA CCT CGA CTA CCC TTG TAA CAT
 F I S I L L W L Y G A D G N I V>

ATG ACC CAA TCT CCC AAA TCC ATG TCC ATG TCA GTA GGA GAG AGG GTC
 TAC TGG GTT AGA GGG TTT AGG TAC AGG TAC AGT CAT CCT CTC TCC CAG
 M T Q S P K S M S M S V G E R V>

ACC TTG ACC TGC AAG GCC AGT GAG
 TGG AAC TGG ACG TTC CGG TCA CTC
 T L T C K A S E>

5 / 30

FIG.5

TTC GAA GCC GCC ACC ATG AGG TGC TCT GCT GAG TTT CTG GGG TTG CTG
 AAG CTT CGG CGG TGG TAC TCC ACG AGA CGA CTC AAA GAC CCC AAC GAC
 M R C S A E F L G L L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG TCT
 GAC GAC ACC GAA TGT CTA CGG TCT ACA CTG TAG GTC TAC TGA GTC AGA
 L L W L T D A R C D I Q M T Q S>

CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT
 GGT CGG AGG GAT AGA CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA
 P A S L S V S V G E T V T I T C>

CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT CGT CAG AAA
 GCT CGT TCA CTC TTA TAA ATG TCA TTA AAT CGT ACC ATA GCA GTC TTT
 R A S E N I Y S N L A W Y R Q K>

CAG GGA AAA TCT CCT CAG CTC CTG GTC TTT GCT GCA TCA AAC TTA GCA
 GTC CCT TTT AGA GGA GTC GAG GAC CAG AAA CGA CGT AGT TTG AAT CGT
 Q G K S P Q L L V F A A S N L A>

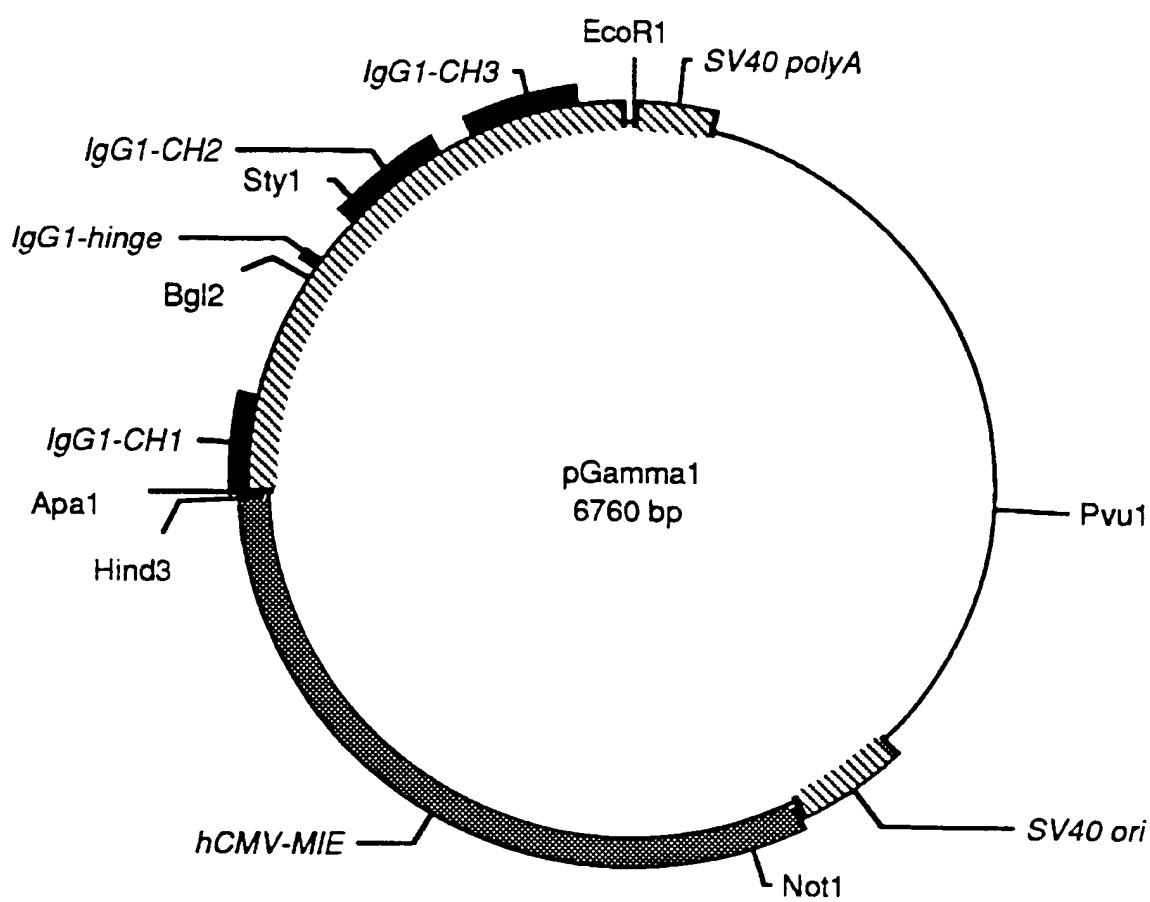
GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TAT
 CTA CCA CAC GGT AGT TCC AAG TCA CCG TCA CCT AGT CCG TGT GTC ATA
 D G V P S R F S G S G S G T Q Y>

TCC CTC AAG ATC AAC AGC CTG CAG TCT GAA GAT TTT GGG GAT TAT TAC
 AGG GAG TTC TAG TTG TCG GAC GTC AGA CTT CTA AAA CCC CTA ATA ATG
 S L K I N S L Q S E D F G D Y Y>

TGT CAA CAT TTT TGG ACT ACT CCG TGG GCG TTC GGT GGA GGC ACC AAC
 ACA GTT GTA AAA ACC TGA TGA GGC ACC CGC AAG CCA CCT CCG TGG TTG
 C Q H F W T T P W A F G G G T N>

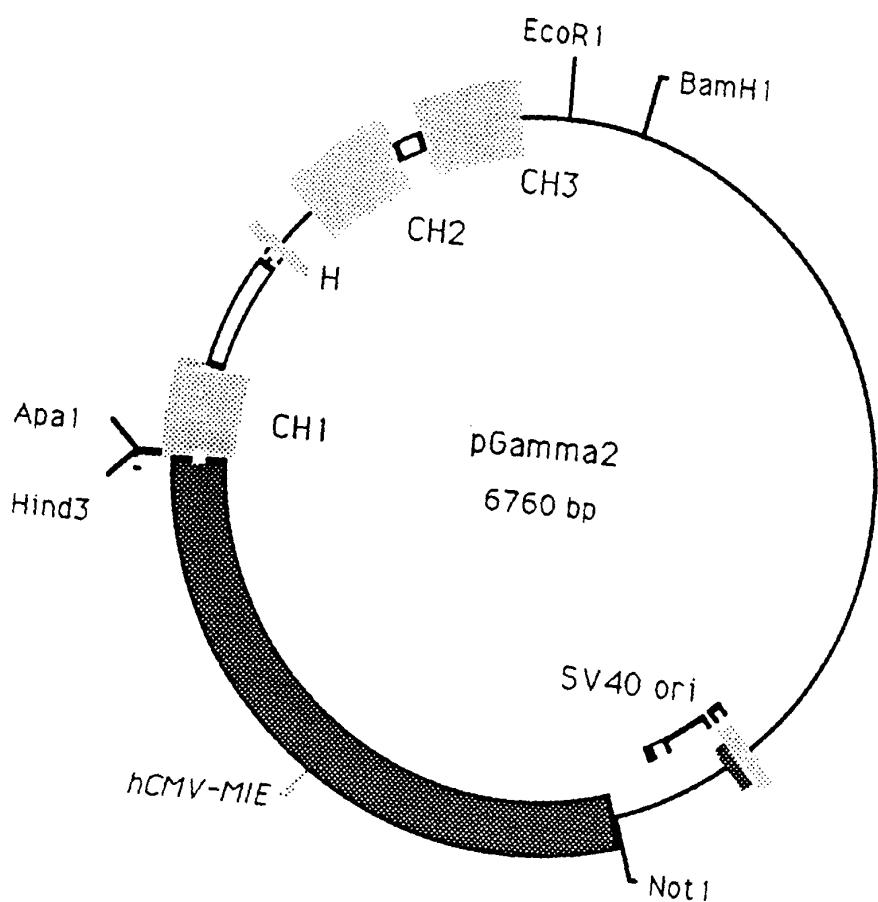
CTG GAA ATC AAA CGT
 GAC CTT TAG TTT GCA
 L E I K R>

FIG. 6



7 / 30

FIG. 7



SUBSTITUTE SHEET (RULE 26)

8 / 30

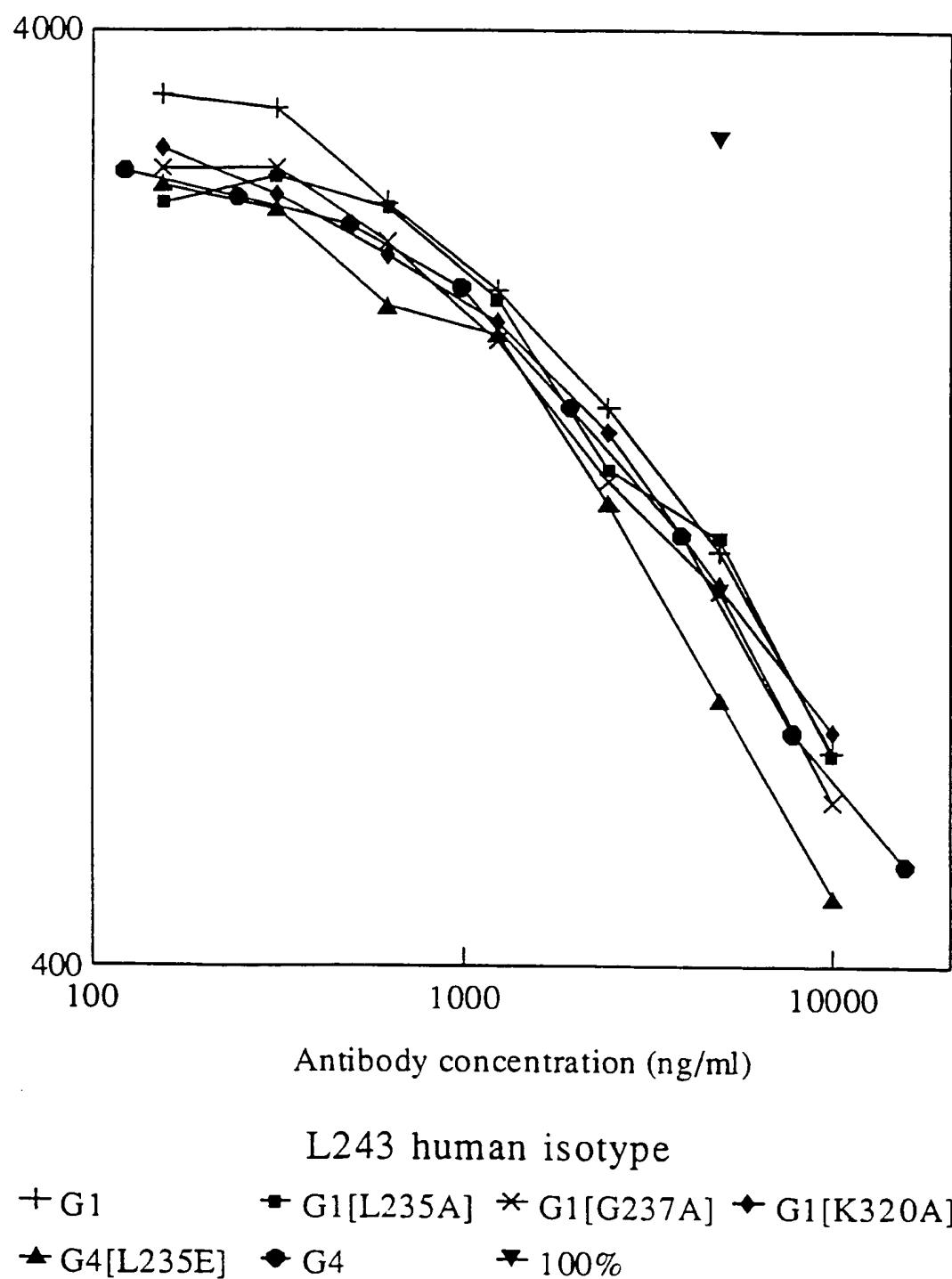
FIG. 8

810	820	830	840	850	860
*	*	*	*	*	*
ACCCC AAAGG CCAAA CTCTC CACTC CCTCA GCTCG GACAC CTCTC CTCCT CCCAG ATCTG					
TGGGG TTTCC GGTTT GAGAG GTGAG GGAGT CGAGC CTGTG GAAGA GAGGA GGGTC TAGAC					
870	880	890	900	910	920
*	*	*	*	*	*
AGTAA CTCCC AATCT TCTCT CTGCA GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA					
TCATT GAGGG TTAGA AGAGA GACGT CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT					
		E P K S C D K T H T>			
930	940	950	960	970	
*	*	*	*	*	
TGC CCA CCG TGC CCA GGTAA GCCAG CCCAG GCCTC GCCCT CCAGC TCAAG GCGGG					
ACG GGT GGC ACG GGT CCATT CGGTC GGGTC CGGAG CGGGA GGTG AGTTC CGCCC					
C P P C P>					
980	990	1000	1010	1020	1030
*	*	*	*	*	*
ACAGG TGCCC TAGAG TAGCC TGCAT CCAGG GACAG GCCCC AGCCG GGTGC TGACA CGTCC					
TGTCC ACGGG ATCTC ATCGG ACGTA GGTCC CTGTC CGGGG TCGGC CCACG ACTGT GCAGG					
1040	1050	1060	1070	1080	
*	*	*	*	*	
ACCTC CATCT CTGCC TCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC					
TGGAG GTAGA GAAGG AGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG					
A P E L L G G P S V F L>					
1090	1100	1110	1120	1130	1140
*	*	*	*	*	*
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA					
AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT					
F P P K P K D T L M I S R T P E V T>					
1150	1160	1170	1180	1190	
*	*	*	*	*	
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC					
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG					
C V V V D V S H E D P E V K F N W Y>					
1200	1210	1220	1230	1240	1250
*	*	*	*	*	*
GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC					
CAC CTG CCG CAC CTC CAC GCA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG					
V D G V E V H N A K T K P R E E Q Y>					

9 / 30

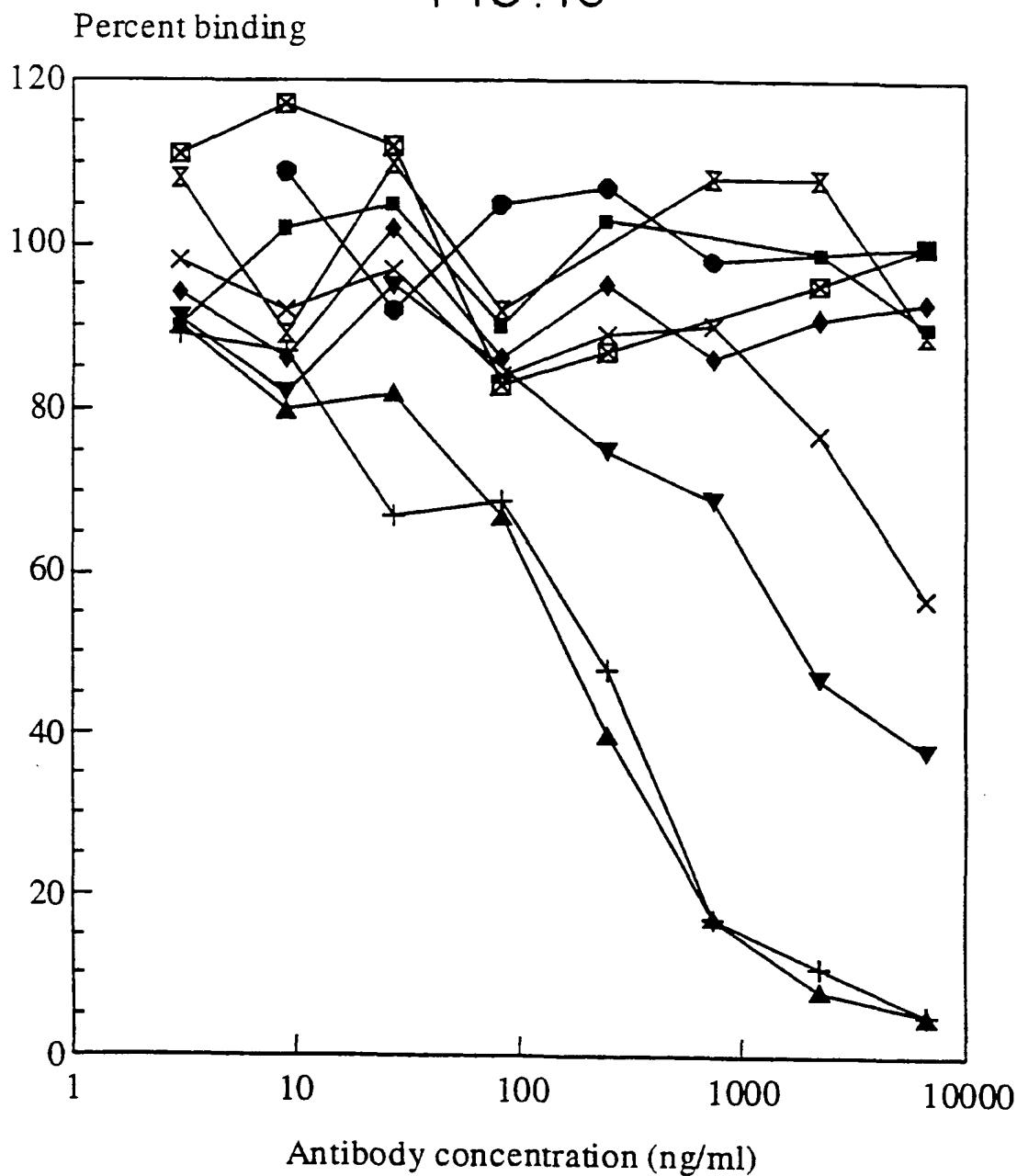
FIG. 9

MFI on JY cells



10 / 30

FIG. 10



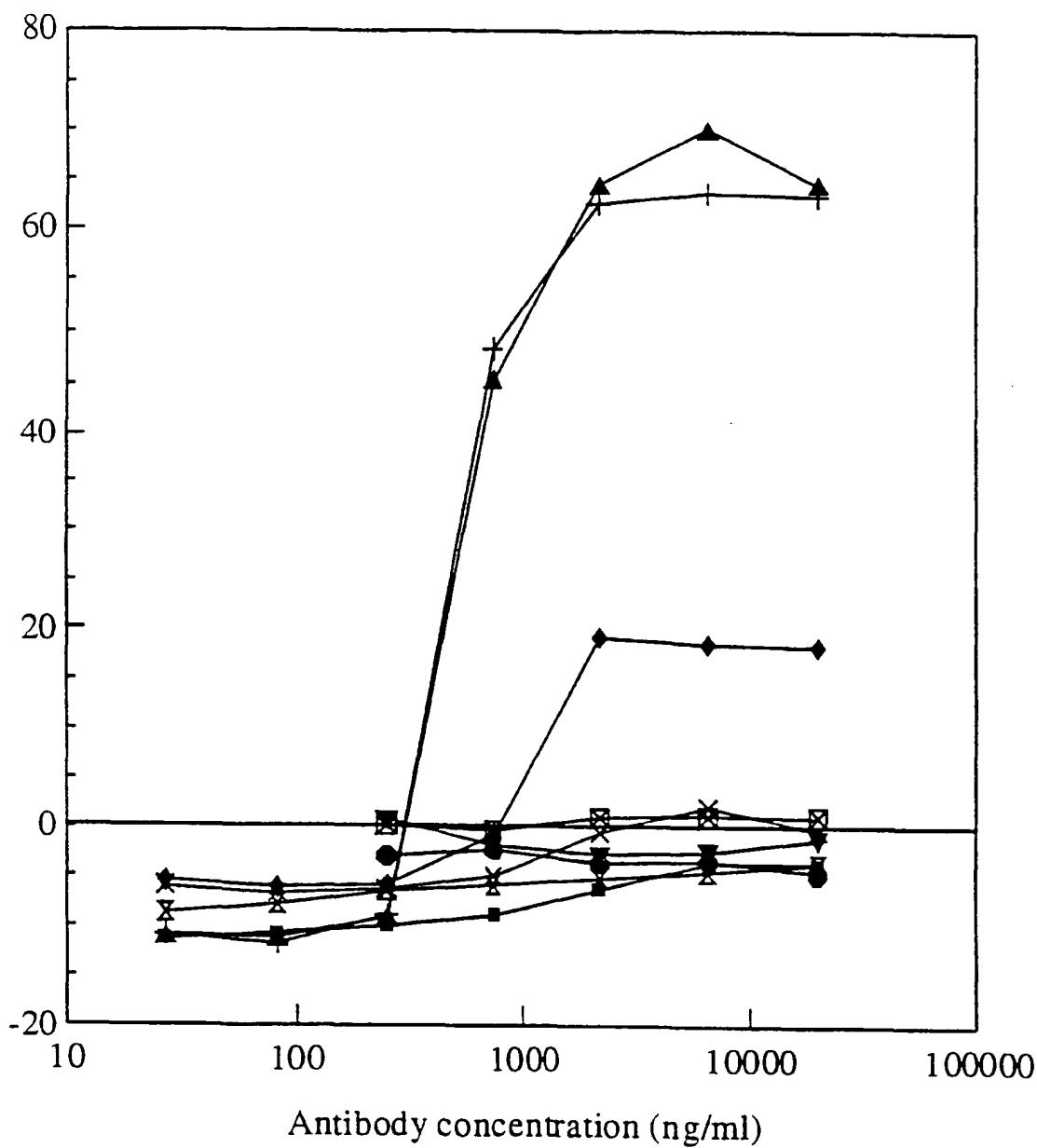
L243 isotype

- + G1 ■ G1[L235E] ✕ G1[L235A]
- ◆ G1[G237A] ★ G1[K320A] ✗ G1/G2L hinge
- G2 ▾ G4 ☐ G4[L235E]

11 / 30

FIG. 11

Percent JY cells killed



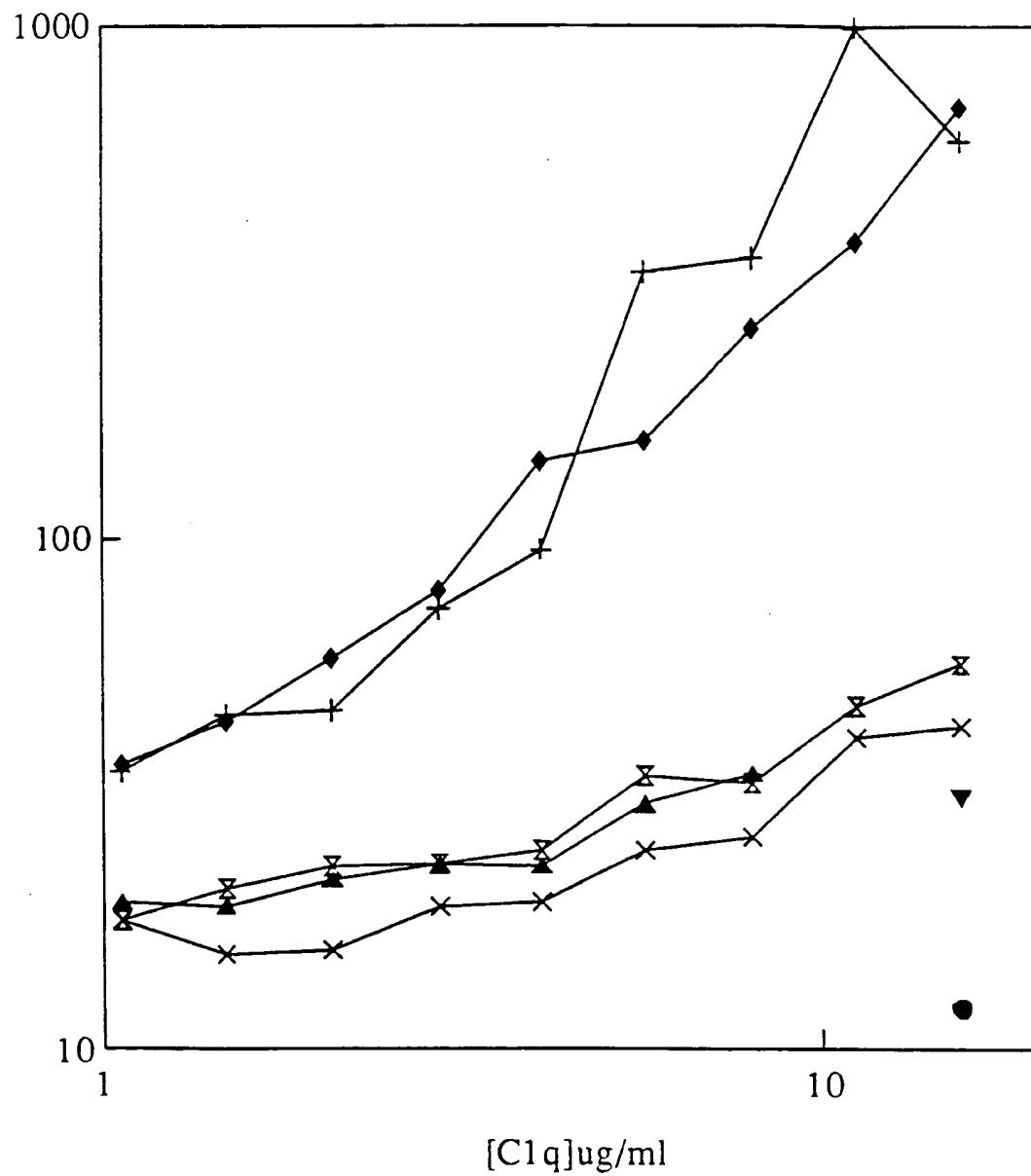
L243 isotype

- | | | |
|-------------|-------------|---------------|
| + G1 | ■ G1[L235E] | * G1[L235A] |
| ◆ G1[G237A] | ★ G1[K320A] | ✖ G1/G2Lhinge |
| ● G2 | ▼ G4 | ☒ G4[L235E] |

12 / 30

FIG. 12

Median FL1 on JY cells



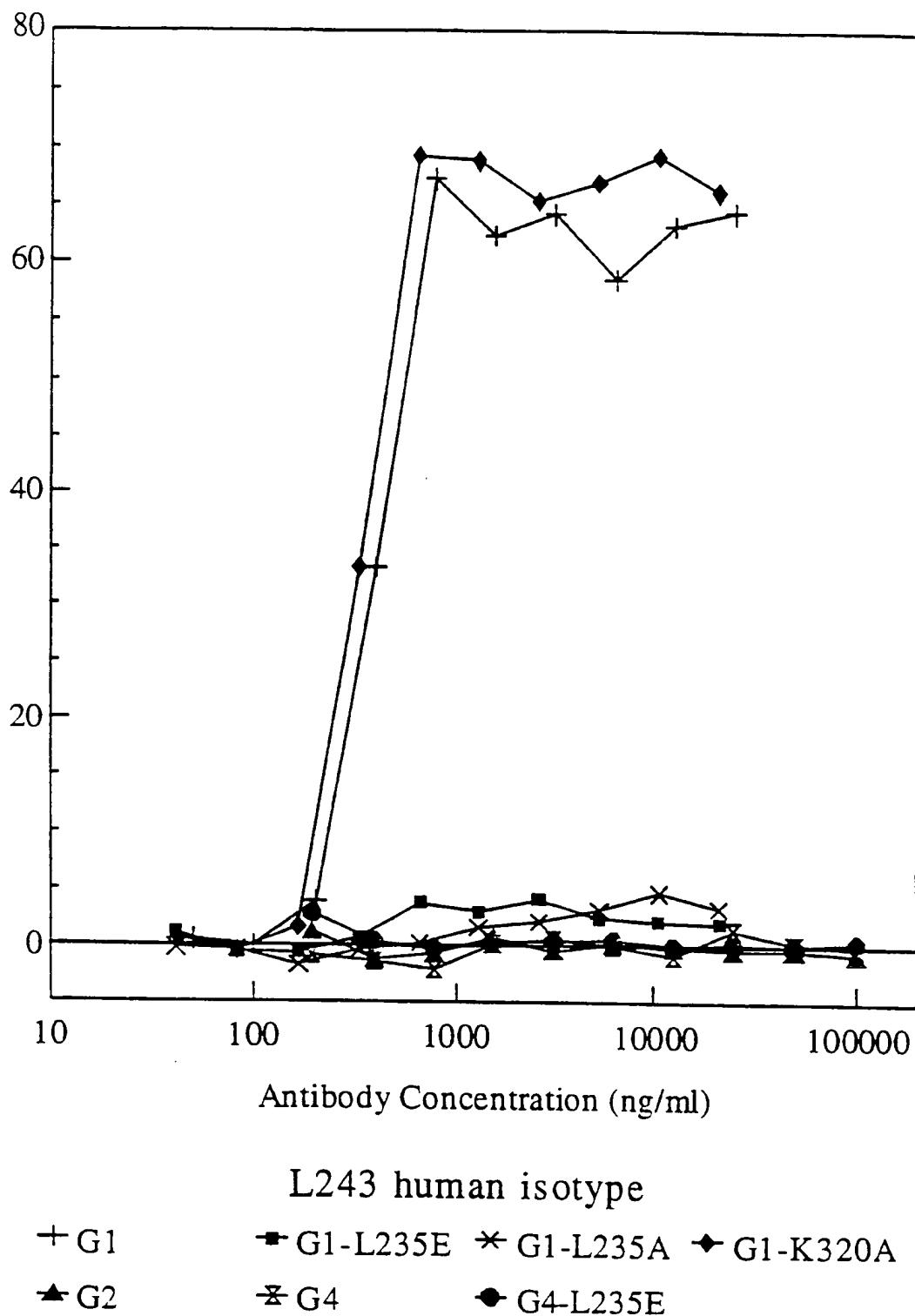
L243 human isotype

- + G1 * G1[L235E] ♦ G1[K320A] ★ G4
- ※ G4[L235E] ● Cells alone ▲ Cells + C1q

13 / 30

FIG. 13

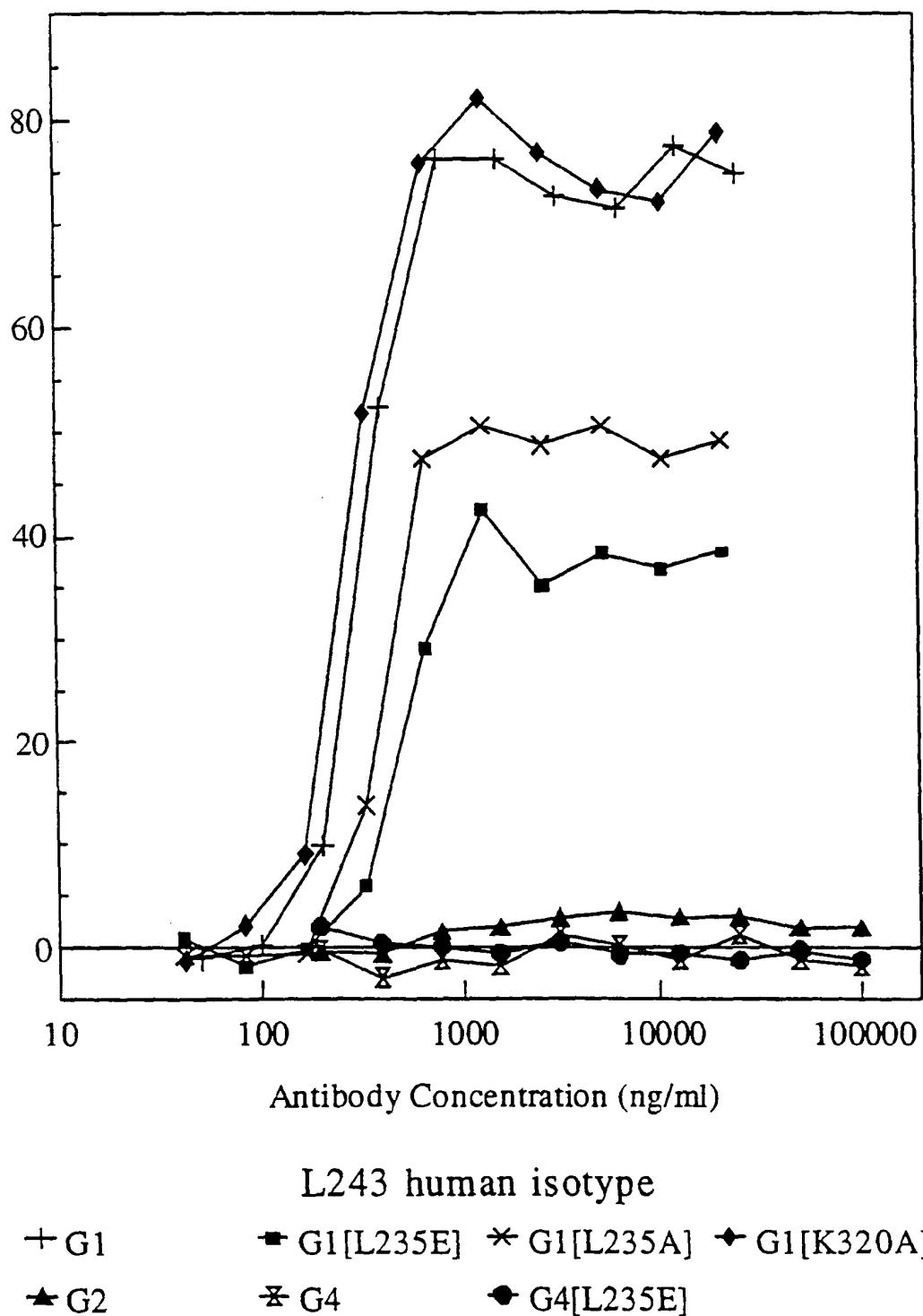
Percent JY cells killed



14 / 30

FIG. 14

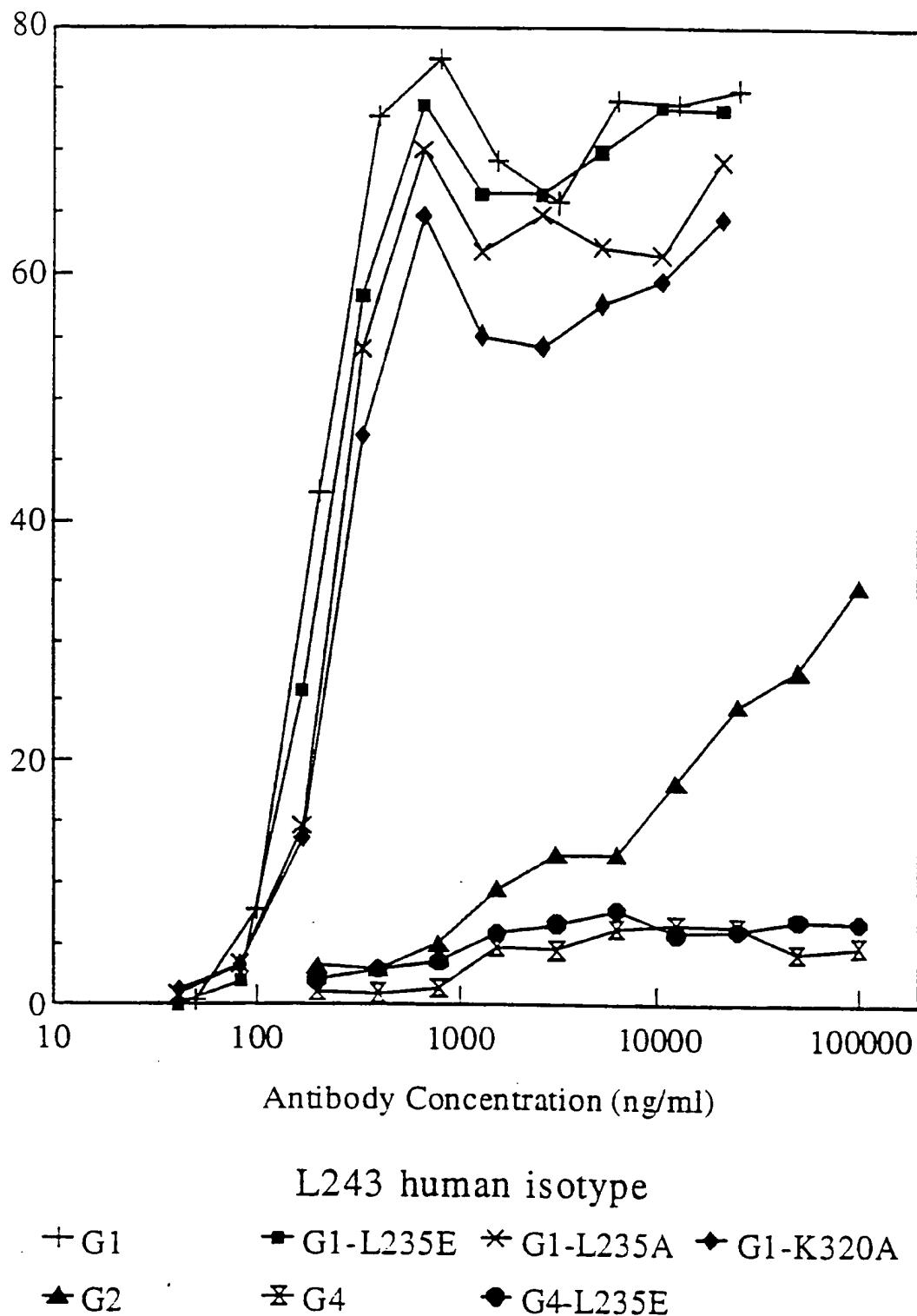
Percent JY cells killed



15 / 30

FIG. 15

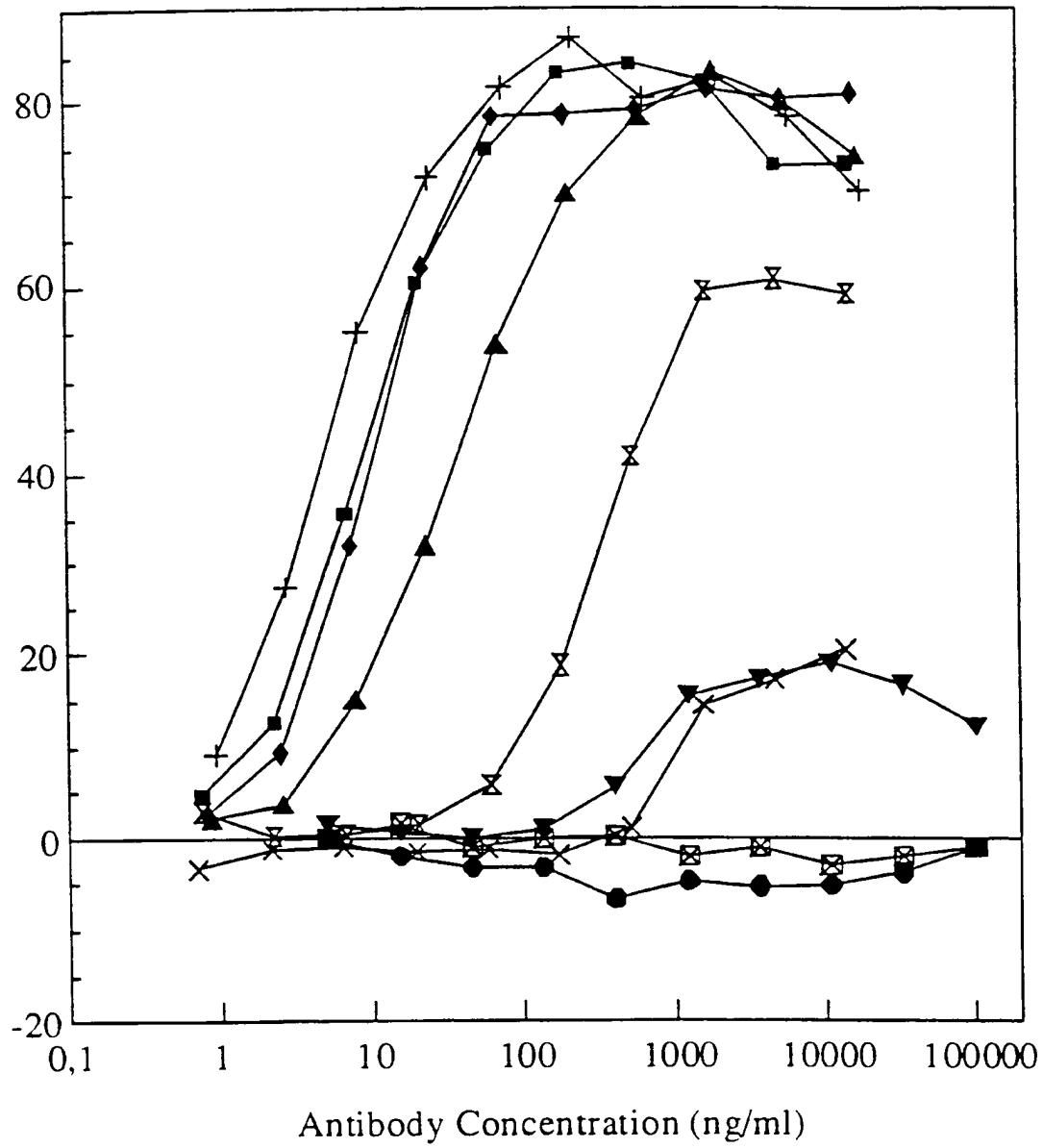
Percent JY cells killed



16 / 30

FIG. 16

Percent JY cells killed

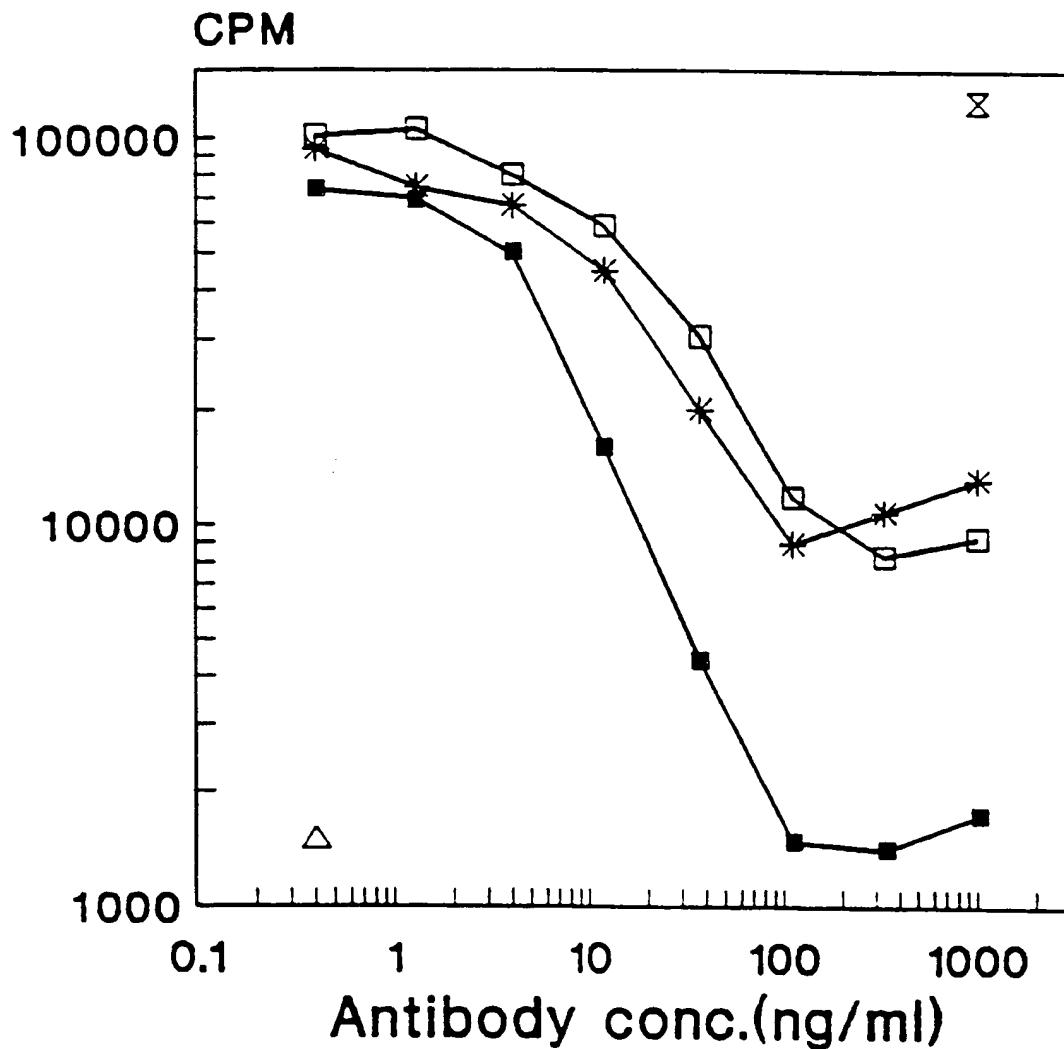


L243 human isotype

- + G1
- ◆ G1[K320A]
- ▲ G1[L235E]
- ◆ G1[L235A]
- ★ G1[L235E]
- ◆ G1[G237A]
- ◆ G1/G2L-hinge
- G2
- ▼ G4
- ◆ G4[L235E]

17 / 30

FIG. 17

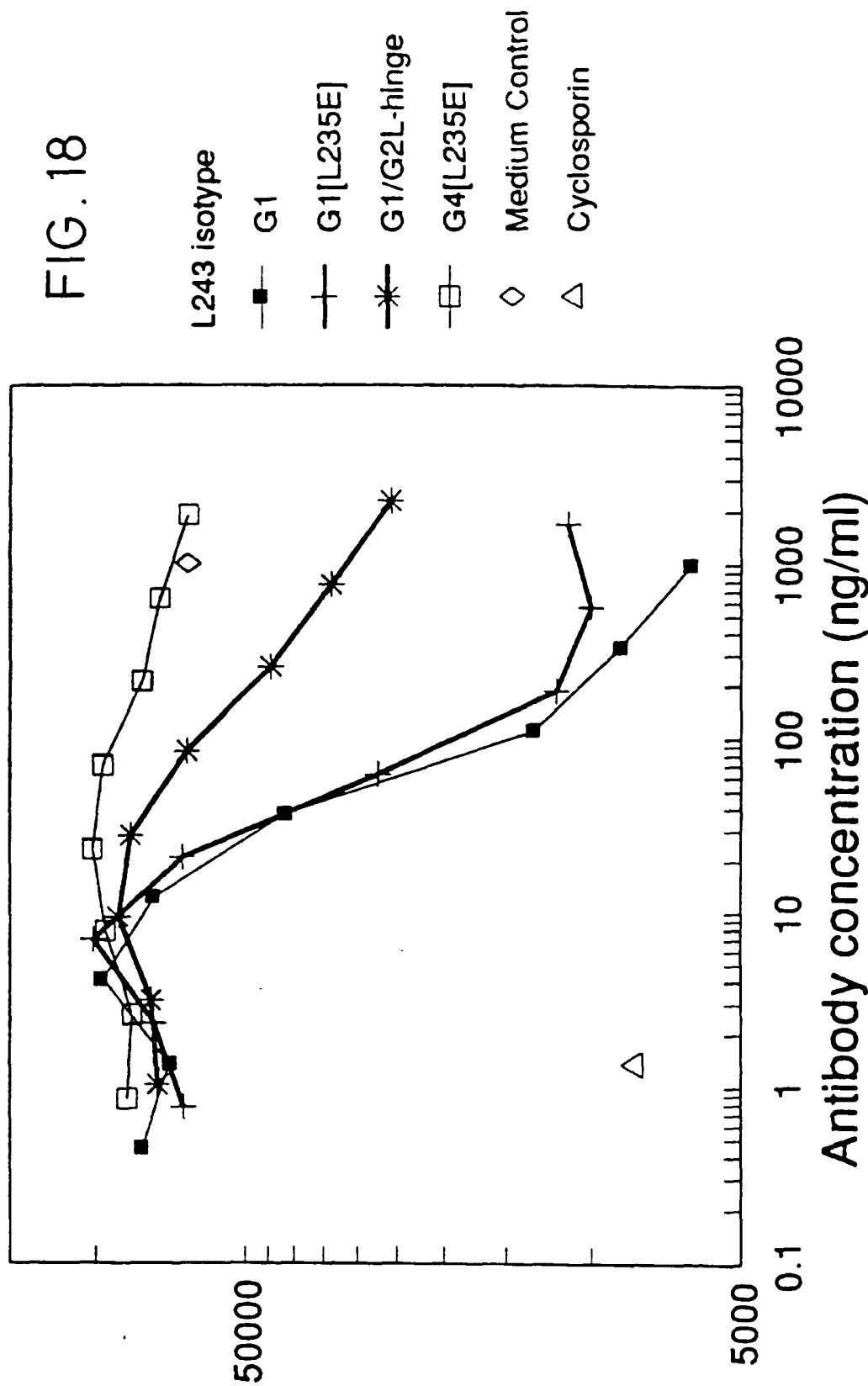


L243 human isotype

- G1
- ＊ G2
- G4
- △ Cyclosporin
- × Medium control

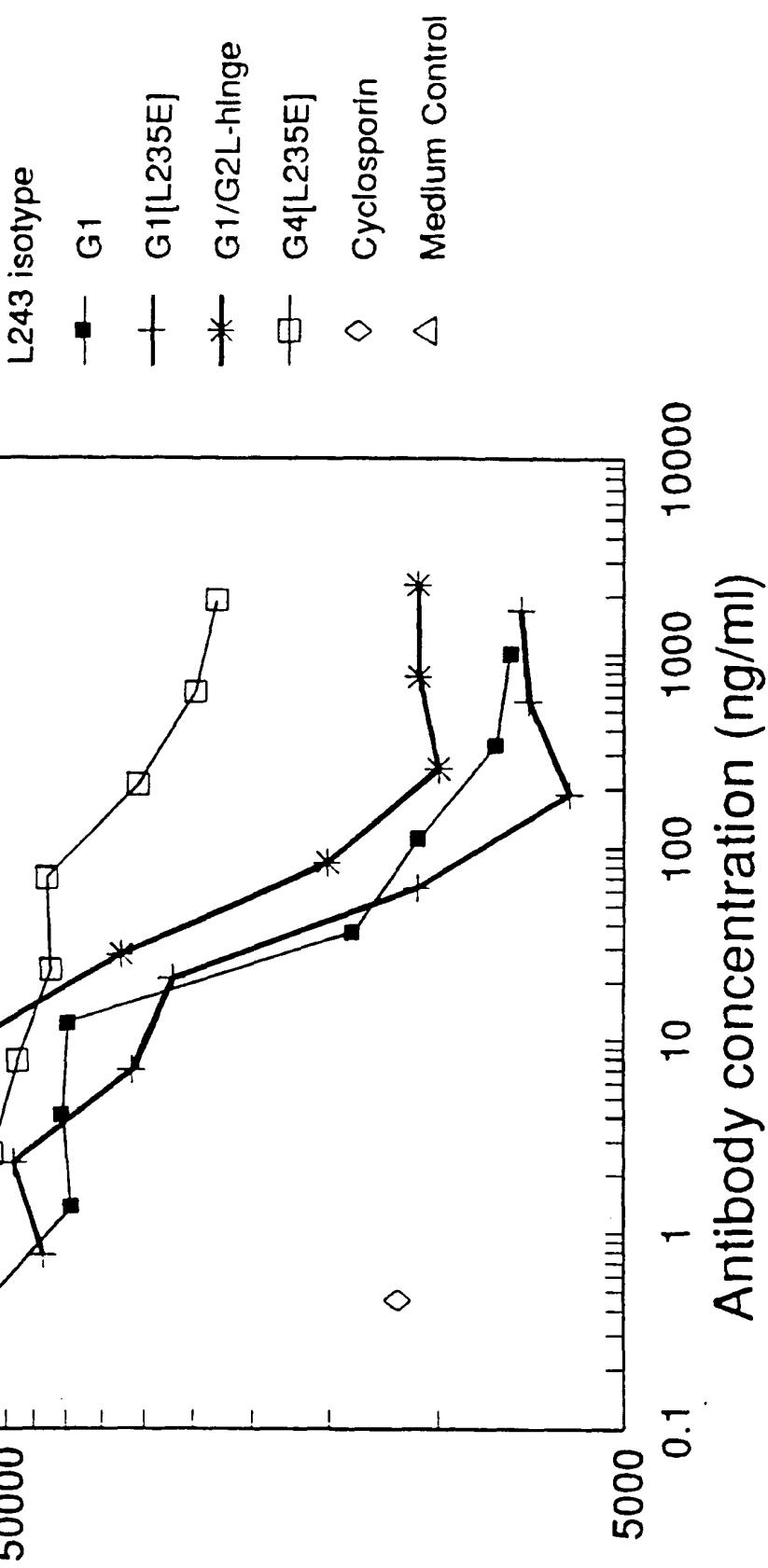
18 / 30

FIG. 18
CPM



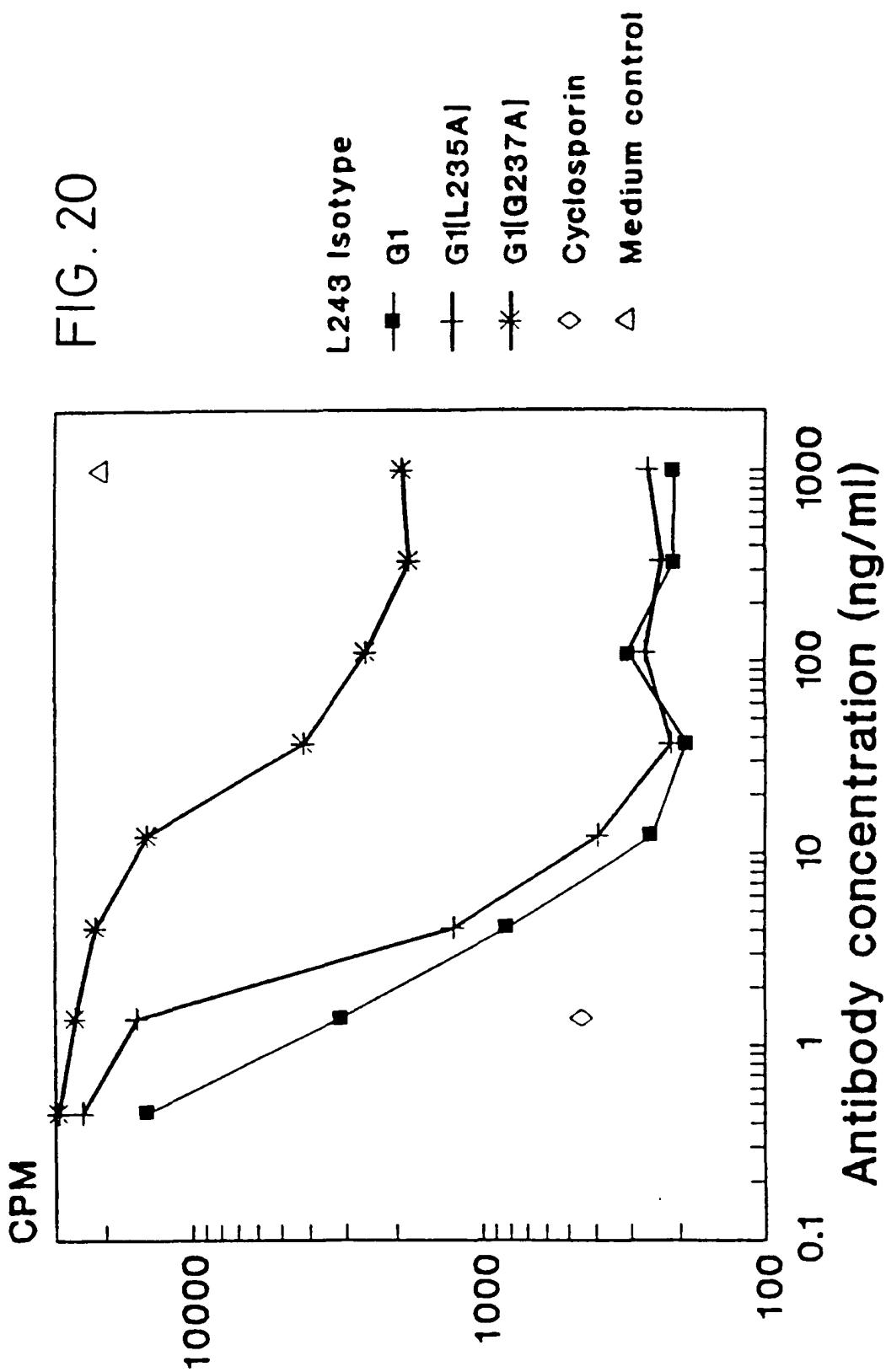
19 / 30

FIG. 19
CPM



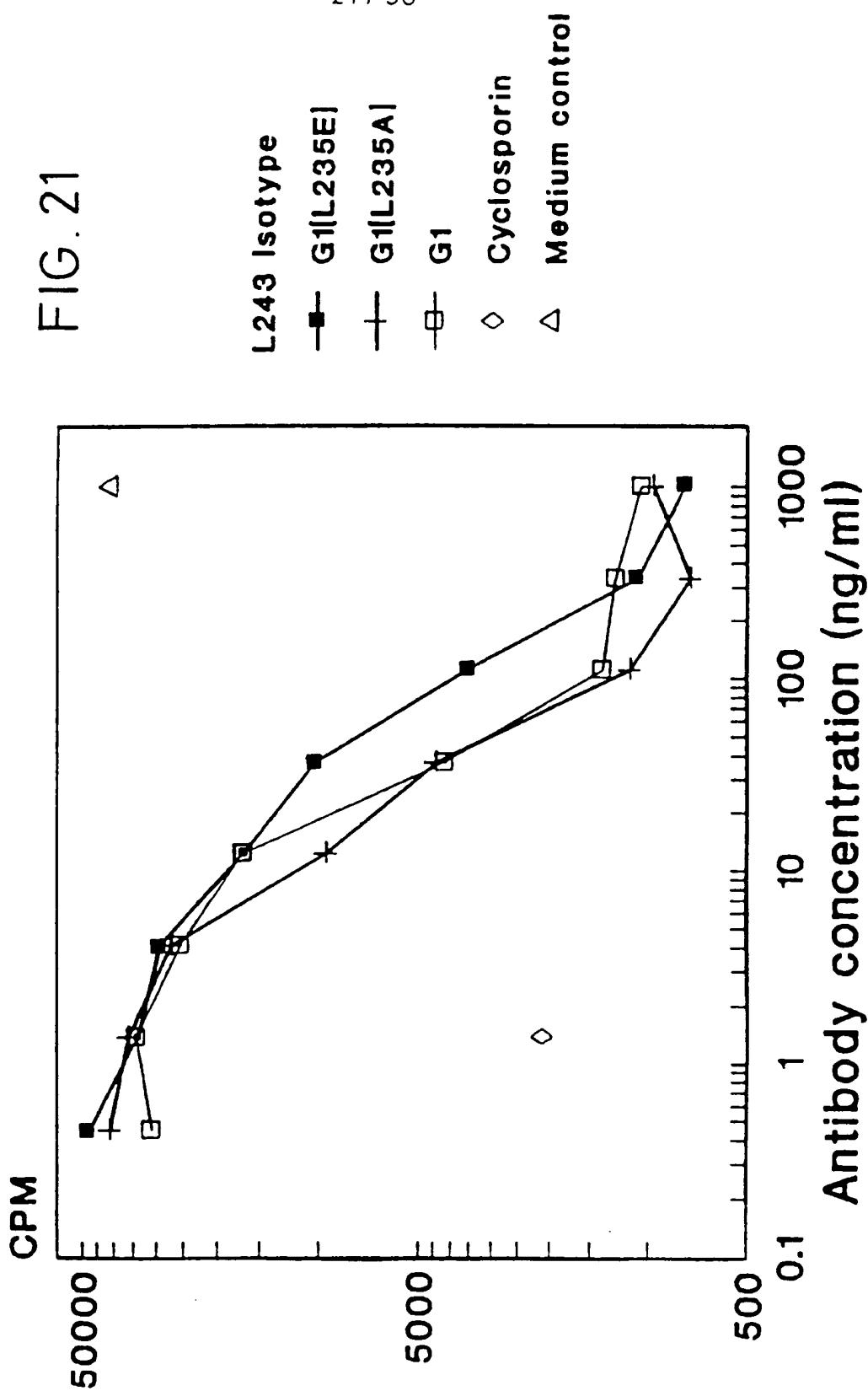
20 / 30

FIG. 20



21 / 30

FIG. 21



22 / 30

FIG. 22

TTCGAAGCCGCCACC ATG TCT GTC CCC ACC CAA GTC CTC GGT CTC CTG
 M S V P T Q V L G L L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATT CAA ATG ACC CAG
 L L W L T D A R C D I Q M T Q>

AGC CCA TCC AGC CTG AGC GCA TCT GTA GGA GAC CGG GTC ACC ATC
 S P S S L S A S V G D R V T I>

ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT
 T C R A S E N I Y S N L A W Y>

CAG CAG AAA CCA GGA AAA GCT CCT CAG CTC CTG ATC TTT GCT GCA
 Q Q K P G K A P Q L L I F A A>

TCA AAC TTA GCA GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA
 S N L A D G V P S R F S G S G>

TCA GGC ACA CAG TAT ACC CTA ACT ATC TCC AGC CTG CAG CCA GAA
 S G T Q Y T L T I S S L Q P E>

GAT TTT GCT ACT TAT TAC TGT CAA CAT TTT TGG ACT ACT CGG TGG
 D F A T Y Y C Q H F W T T P W>

GCG TTC GGT CAA GGC ACC AAA GTG GAA ATC AAA CGT
 A F G Q G T K V E I K R>

23 / 30

FIG. 23

TTGGAAGCGGCCACC ATG TCT GTC CCC ACC CAA GTC CTC GGT CTC CTG
 M S V P T Q V L G L L>

 CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATT CAA ATG ACC CAG
 L L W L T D A R C D I Q M T Q>

 AGC CCA TCC AGC CTG AGC GCA TCT GEA GGA GAC CGG GTC ACC ATC
 S P S S L S A S V G D R V T I>

 ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT
 T C R A S E N I Y S N L A W Y>

 CAG CAG AAA CCA GGA AAA GCT CCT AAG CTC CTG ATC TAT GCT GCA
 Q Q K P G K A P K L L I Y A A>

 TCA AAC TTA GCA GAT GGT GTG CCA TCT AGA TTC AGT GGC AGT GGA
 S N L A D G V P S R F S G S G>

 TCA GGC ACA GAC TTT ACC CTA ACT ATC TCC AGC CTG CAG CCA GAA
 S G T D F T L T I S S L Q P E>

 GAT TTT GCT ACT TAT TAC TGT CAA CAT TTT TGG ACT ACT CGG TGG
 D F A T Y Y C Q H F W T T P W>

 GCG TTC GGT CAA GGC ACC AAA GTG GAA ATC AAA CGT
 A F G Q G T K V E I K R>

24 / 30

FIG. 24

AAGCTTGGCGCCACC ATG GAG TGG TCT TGG GTG TTC CTG TTC TTC CTG
M E W S W V F L F F L>

TCT GTG ACA ACA GGA GTG CAC TCT CAG GTG CAG CTG GTG CAG TCT
S V T T G V H S Q V Q L V Q S>

GGA GCA GAG GTG AAG AAG CCT GGA GCA TCT GTG AAG GTG TCT TGT
G A E V K K P G A S V K V S C>

AAG GCA TCT GGA TTC ACA TTC ACA AAT TAC GGA ATG AAT TGG GTG
K A S G F T F T N Y G M N W V>

AGA CAG GCA CCT GGA CAG GGA CTC GAG TGG ATG GGA TGG ATT AAT
R Q A P G Q G L E W M G W I N>

ACA TAC ACA AGA GAG CCT ACG TAC GCA GAC GAC TTC AAG GGA AGA
T Y T R E P T Y A D D F K G R>

TTC ACA TTC ACA CTG GAG ACA TCT GCA TCT ACA GCA TAC ATG GAG
F T F T L E T S A S T A Y M E>

CIG TCT TCT CTG AGA TCT GAG GAC ACA GCA GTG TAC TAC TGT GCA
L S S L R S E D T A V Y Y C A>

AGA GAC ATT ACA GCA GTG GTA CCT ACA GGA TTC GAC TAC TGG GGA
R D I T A V V P T G F D Y W G>

CAG GGA ACA CTG GTG ACA GTG TCT TCT
Q G T L V T V S S>

25 / 30

FIG. 25

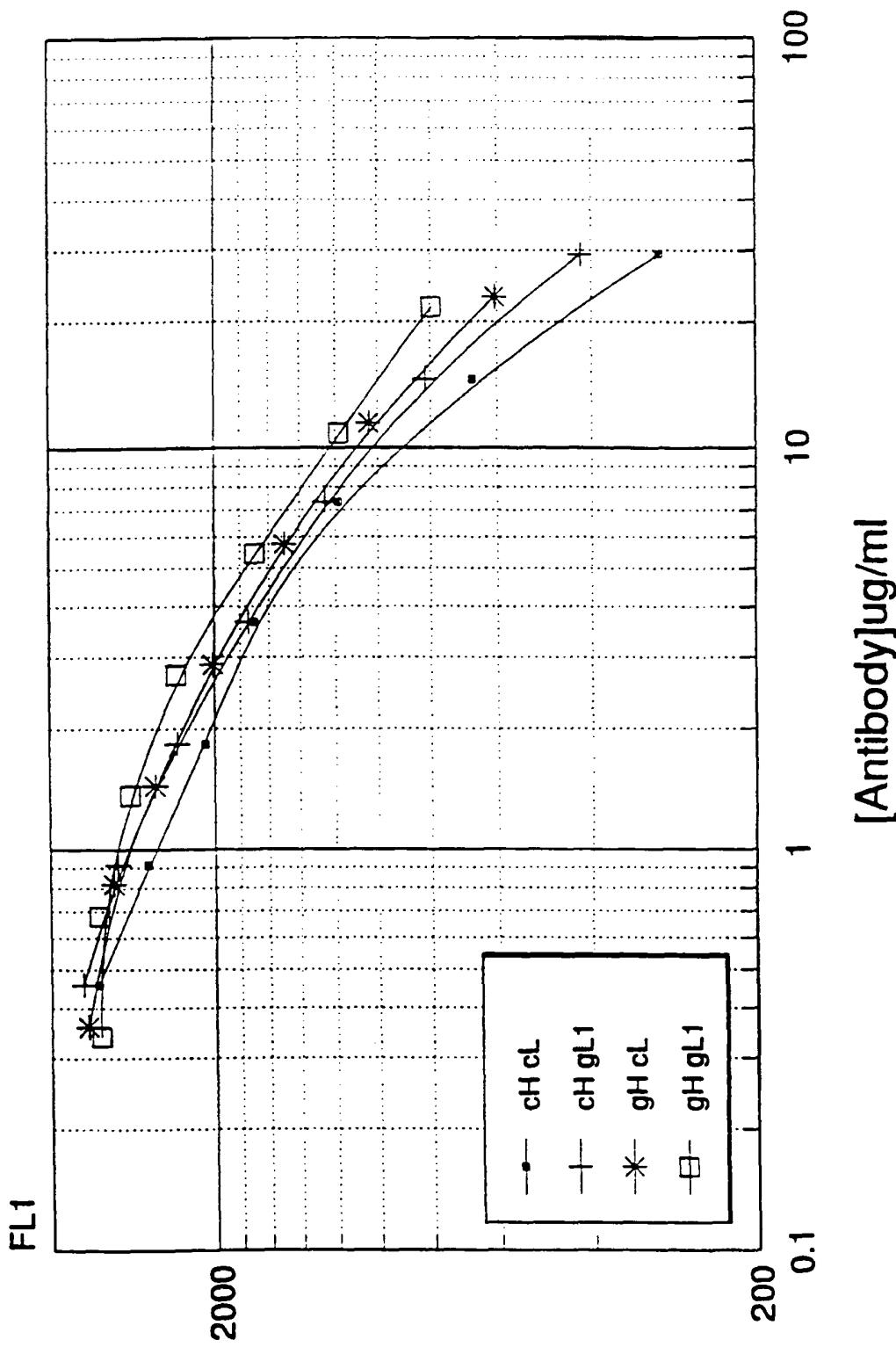
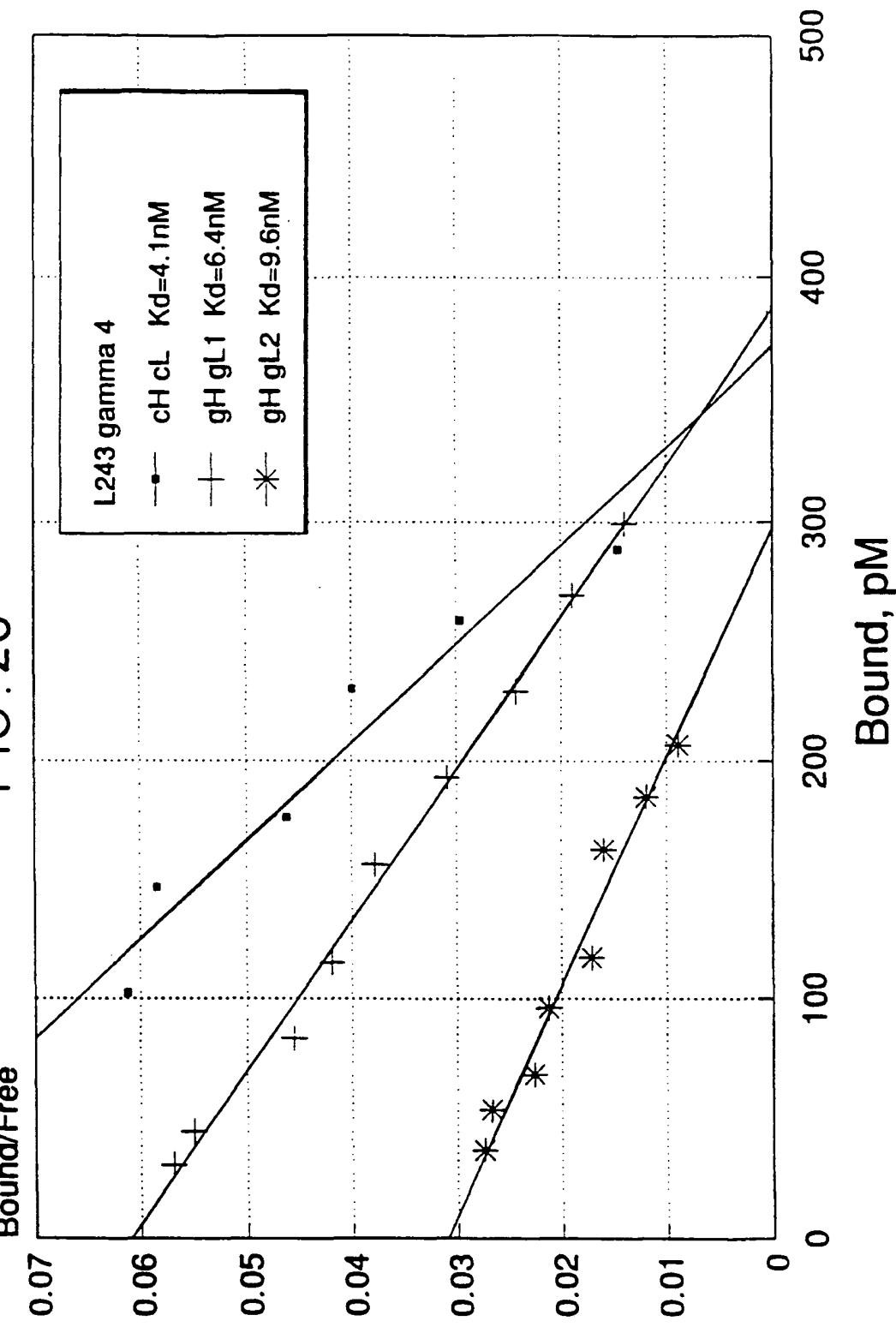


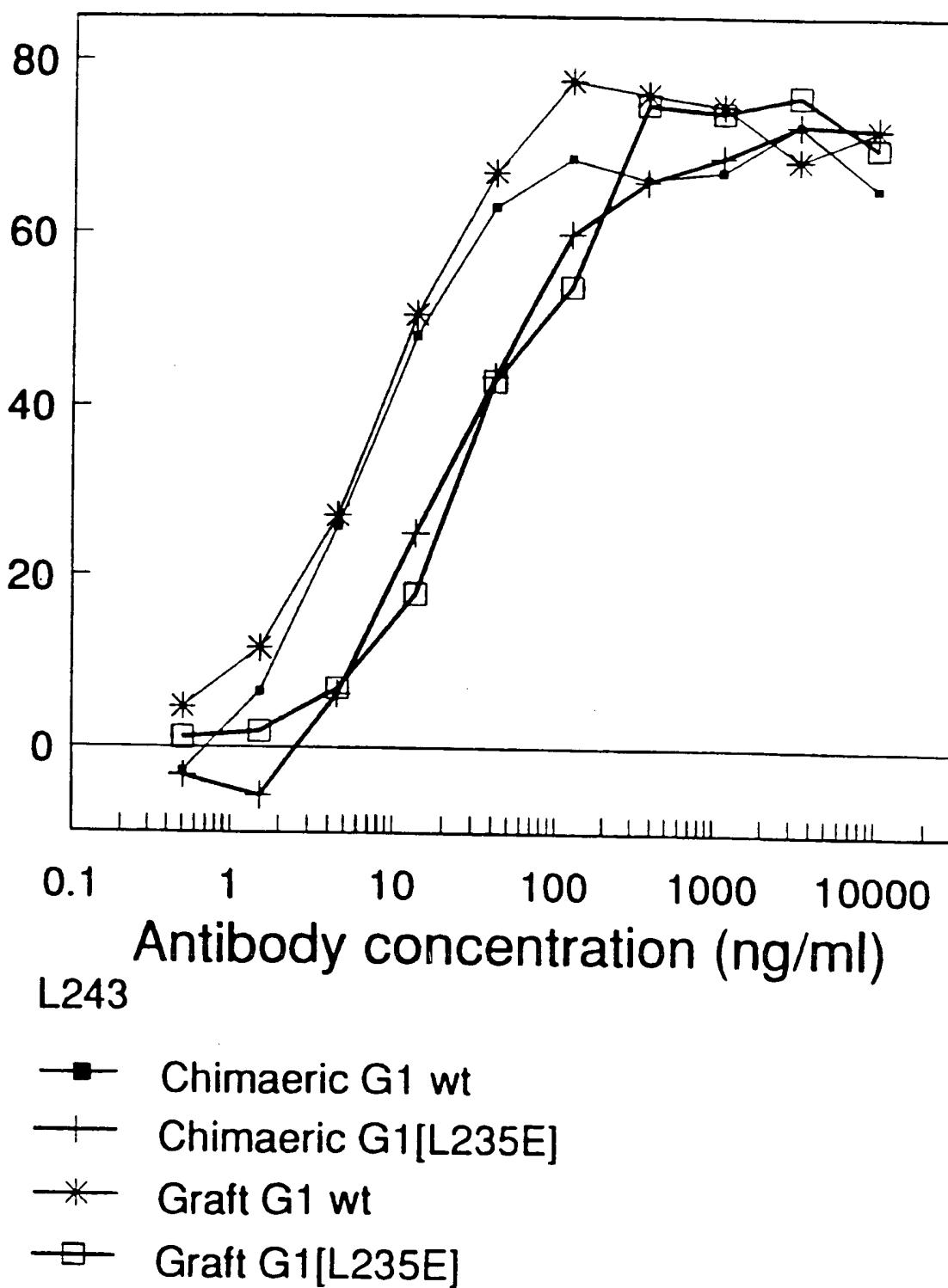
FIG. 26



27 / 30

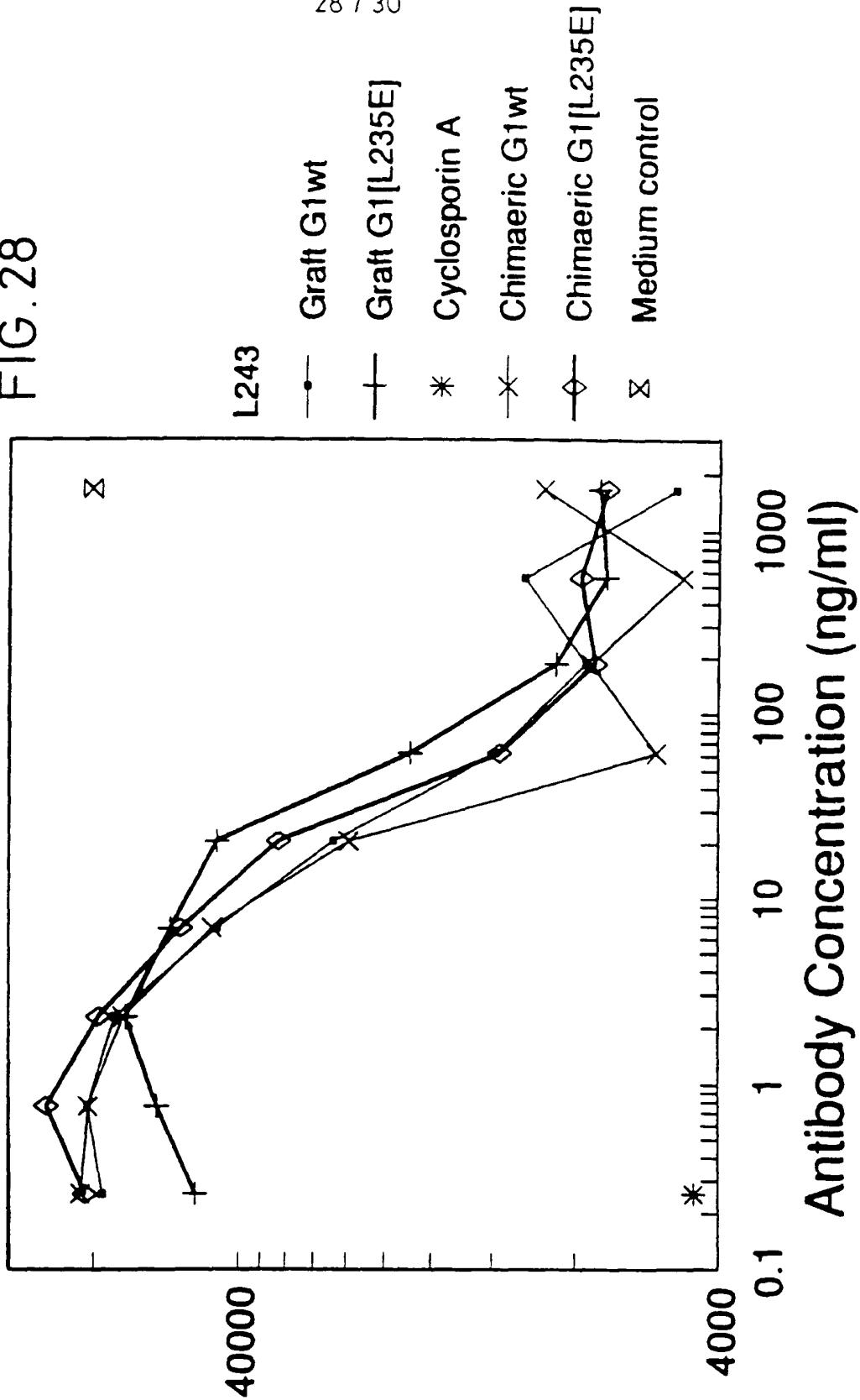
FIG. 27

JY cells killed (%)



28 / 30

FIG. 28
CPM



29 / 30

FIG. 29

